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CHARACTERIZATION OF THE NOVEL ENDONUCLEASE SAE2 INVOLVED IN DNA END PROCESSING

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CHARACTERIZATION OF THE NOVEL ENDONUCLEASE SAE2 INVOLVED IN DNA END PROCESSING

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At the very center of sexual reproduction is meiosis. During meiosis, the formation of meiotic Double-Strand-Breaks (DBSs) and their repair by homologous recombination are widely conserved events occurring among most eukaryote species. Meiosis-specific DSB formation requires at least nine proteins (Spo11, Ski8, Rec102, Rec104, Mei4, Mer2, Rec114, Mre11/Rad50/Xrs2) in *S. cerevisiae*, and the resection of the DSB ends requires additional four proteins (Mre11/Rad50/Xrs2, and Sae2). Spo11 has been identified as the catalytic component of this DSB-initiating complex. However, the roles played by the majority of these proteins are not clear. I have purified the recombinant Spo11/Ski8/Rec102/Rec104 complex, characterized its DNA binding ability as well as its cleavage activity on supercoiled plasmid DNA.

Sae2 functions in both meiotic and mitotic repair of DNA double-strand breaks (DSBs) in *S. cerevisiae*. *In vivo* experiments have shown that Sae2 collaborates with the Mre11/Rad50/Xrs2 (MRX) complex in DNA end processing. Our laboratory previously showed that recombinant Sae2 exhibits endonuclease activity on single-stranded DNA

and single-strand/double-strand DNA junctions using purified proteins *in vitro*. The MRX complex stimulates Sae2 endonuclease activity on single-stranded DNA close to single-strand/double-strand junctions, through its endonucleolytic activity. However, Sae2 contains no conserved typical nuclease domain, and it only shares very limited homology with its human functional counterpart CtIP. To characterize Sae2 and the active sites responsible for its nuclease activity, I used partial proteolysis and site-directed mutagenesis to analyze the protein. Biochemical assays *in vitro* show that acidic residues in the central domain play an important role in Sae2 endonuclease activity. Sae2 has also been shown to be phosphorylated by CDK (Cyclin-Dependent Kinase) during the S and G_2 phases of the cell cycle, as well as by Tel1/Mec1 upon DNA damage. These modifications are essential for the function of Sae2 in DNA repair, but the function of these modifications are not clear. I have demonstrated that, in the presence of MRX, Sae2 (5D/S267E) mimicking constitutive phosphorylation by CDK and Mec1/Tel1 can assist the 5' to 3' exonuclease Exo1 significantly in 5' end resection by suppressing the inhibitory effect of Ku. These results suggest that Sae2 is a critical switching protein which determines the choice between HR and NHEJ in yeast cells upon DNA damage.

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CHAPTER 1: INTRODUCTION

MEIOSIS-SPECIFIC DNA DOUBLE-STRAND BREAKS

In most sexually reproducing organisms, the formation and repair of double-strand breaks is essential for proper gamete formation. The process of meiotic recombination, which proceeds via the formation and repair of meiotic DSBs, forms physical connections between homologous chromosomes. These connections are critical for the proper orientation of homologous chromosomes on the spindle and their proper segregation at the first division afterwards. If recombination fails or does not occur, meiosis will produce aneuploid and inviable gametes. In budding yeast, and in other organisms, meiotic recombination initiates at the sites of meiotic-specific DNA DSBs (Keeney 2001).

The frequency of meiotic recombination is strikingly high in comparison with that of spontaneous mitotic recombination. In *Saccharmyces cerevisiae*, for example, the recombination rate during vegetative growth is about $10^{-6} – 10^{-7}$ per locus per generation (Steele, Morris et al. 1991; Keeney 2001). In contrast, the frequency during meiosis is between $10^{-2} – 10^{-1}$ per locus per generation, which represents an increase of 10^{5} fold or more. This increase results from a highly regulated pathway which starts from Spo11-catalyzed programmed DNA DSB formation.

SPO11 AS THE CATALYST FOR MEIOTIC DSBS

In budding yeast, *SPO11* was first identified in a screen for temperature-sensitive mutants that failed in ascospore formation (Esposito, Frink et al. 1972; Esposito and Esposito 1974). *SPO11* encodes a 45-kDa protein, and its expression is controlled at the transcriptional and post-transcriptional levels, so that Spo11 is only expressed during

meiosis. The eukaryotic members of Spo11 all share sequence similarity with the smaller A subunit of TopoVI (Top6A), a type II topoisomerase from archaea (Ajimura, Leem et al. 1993; Bergerat, de Massy et al. 1997; Keeney, Giroux et al. 1997). The topoisomerase activity of archeal type II topoisomerase has been directly demonstrated for Topo VI from *Sulfolobus shibatae* (Bergerat, Gadelle et al. 1994; Buhler, Gadelle et al. 1998). It exhibits an ATP-dependent decatenating and relaxing activity towards double-stranded DNA that involves a transient covalent bond between protein and DNA. Topo VI is an A2B2 heterotetramer, the A subunit of which (Top6A) can bind DNA nonspecifically (Nichols, DeAngelis et al. 1999). However, it does not efficiently cleave DNA by itself (Buhler, Gadelle et al. 1998). The B subunit of Topo VI contains an ATP-binding motif (Bergerat, de Massy et al. 1997). In typical type II topoisomerases, ATP binding and hydrolysis in the B subunit is essential to drive conformational changes which lead to the capture of a second DNA strand, and control the opening and closing of the DNA gate during strand transfer.

Based on the catalytic mechanism of type II topoisomerases, Spo11 is thought to create DSBs by forming dimers to coordinately break both strands of a DNA molecule. The catalytic tyrosine residue in each Spo11 monomer forms a covalent 5'-phosphodiester linkage with the newly created 5' DNA end (Figure 1.1), demonstrated by the findings that Spo11-DNA covalent complexes accumulate in *rad50S* and *Δsae2* strains (Neale, Pan et al. 2005; Keeney and Neale 2006). In wild-type *S. cerevisiae*, these intermediates appear just transiently, and then are further processed via asymmetric endonucleolytic cleavage that is likely mediated by Mre11/Rad50/Xrs2 (MRX), and Sae2 (Cao, Alani et al. 1990; McKee and Kleckner 1997; Prinz, Amon et al. 1997; Moreau, Ferguson et al. 1999) (Figure 1.1). Spo11 homologs in fission yeast (Lin and Smith

1994), plants (Hartung and Puchta 2000; Grelon, Vezon et al. 2001), worms (Dernburg, McDonald et al. 1998; Dernburg, Zalevsky et al. 2000), flies (McKim, Green-Marroquin et al. 1998; McKim and Hayashi-Hagihara 1998), multicellular fungi (Celerin, Merino et al. 2000) and mammals (Baudat, Manova et al. 2000; Romanienko and Camerini-Otero 2000) have also been shown to play an essential role in meiotic DNA DSBs initiation, similar to budding yeast. Spo11 from different eukaryotic species shares higher conservation among two structural domains over the entire length of the protein. The most conserved structural domain is named the "5Y-CAP" motif, since it is shared by all topoisomerases that generate a covalent 5'-tyrosyl phosphodiester bond (Berger 1998). This motif is an α-helical fold similar to the *E. coli* CAP (catabolite gene activator protein) DNA binding domain. This domain contains only one conserved tyrosine residue, mutation of which confers a null phenotype for meiotic recombination (Y135F in budding yeast) (Bergerat, de Massy et al. 1997).

In spite of the high similarity of Spo11 among different eukaryotic species, and with archaeal Top6A, an obvious homolog of Top6B is not present in eukaryotic genomes (The only exception to date is *A. thaliana*). It is possible that a functional counterpart of Top6B exists but has diverged very significantly from it. The proteins that are essential for meiotic DSB initiation and interacting with Spo11 will be discussed in more detail in later sections. Another possibility is that Spo11 can cleave DNA but does not religate the ends like classical type II topoisomerases, thus it does not require an ATP-binding subunit.

OTHER MEIOSIS-SPECIFIC DNA DSB FORMATION PROTEINS

Spo11 is critical for DSB formation in meiosis, but it does not act alone. From fungi to mammals, many genes have been identified which, when mutated, create

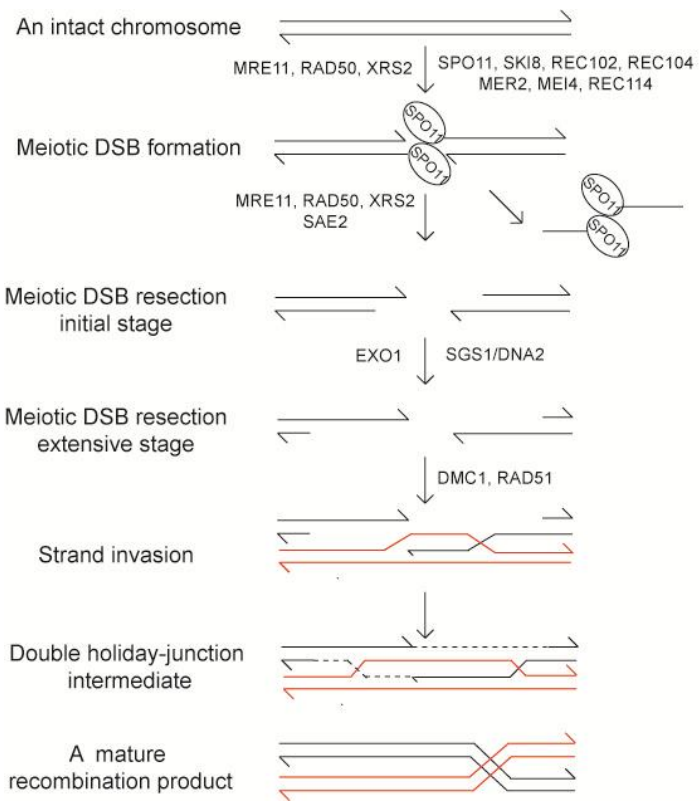


Figure 1.1 Models for Meiotic DSB formation and repair.

During meiosis, at least ten proteins (Spo11, Ski8, Rec102, Rec104, Mei4, Mer2, Rec114, and Mre11/Rad50/Xrs2) are required for the formation of DSBs. Spo11 is the catalytic enzyme in this process. MRX complex and Sae2 are required for the release of Spo11-DNA complex from the chromosomes, and the initiation of resection at DSB ends. Exo1 or Sgs1/Dna2 participates in the extensive resection, which generates long 3' overhangs. The 3' overhangs undergo strand-invasion into an intact homologous chromosome, then generate double-holiday junction intermediates, and finally mature recombination products. (Figure adapted from (Keeney 2001))

recombination defects similar to those of *spo11* null mutants (Huynh, Leblon et al. 1986; McKim, Green-Marroquin et al. 1998; Sekelsky, McKim et al. 1999; Gerecke and Zolan 2000; Merino, Cummings et al. 2000; Chin and Villeneuve 2001; Libby, De La Fuente et al. 2002; Tesse, Storlazzi et al. 2003). In *S. cerevisiae*, the list of Spo11 potential partners is likely the most complete, where Spo11 cleaves with the help of at least nine other proteins: Ski8, Rec102, Rec104, Mer2, Rec114, Mei4, and Mre11/Xrs2/Rad50 (Keeney 2001).

Scott Keeney's laboratory performed a systematic analysis of interactions involving Spo11 and other factors essential for DSB formation in *S. cerevisiae*. Their work, together with immunoprecipitation and co-localization studies from other groups (Salem, Walter et al. 1999; Kee and Keeney 2002; Jiao, Salem et al. 2003; Kee, Protacio et al. 2004; Prieler, Penkner et al. 2005), defines a network of interactions that connects all the DSB related proteins to one another (Figure 1.4).

Importantly, their analysis revealed that Ski8 is most likely the direct partner of Spo11. Ski8 was previously known to inhibit the translation of nonpolyadenylated RNA and take part in 3' to 5' exonucleolytic RNA degradation (Masison, Blanc et al. 1995; Anderson and Parker 1998; van Hoof, Staples et al. 2000; Araki, Takahashi et al. 2001). It was surprising when Ski8 was first found to play a role in meiotic DNA DSB formation (Uetz, Giot et al. 2000), because Ski8 was thought to be a cytoplasmic protein functioning in mitotic cells. It was named *REC103* then, and identified in the same genetic screen together with *REC102*, *REC104*, and *REC114* (Malone, Bullard et al. 1991; Gardiner, Bullard et al. 1997). *REC103* is actually identical to *SKI8*. However, Keeney's group determined that the role of Ski8 in RNA metabolism is genetically separable from its roles in DSB formation. During meiotic prophase I, Ski8 relocates

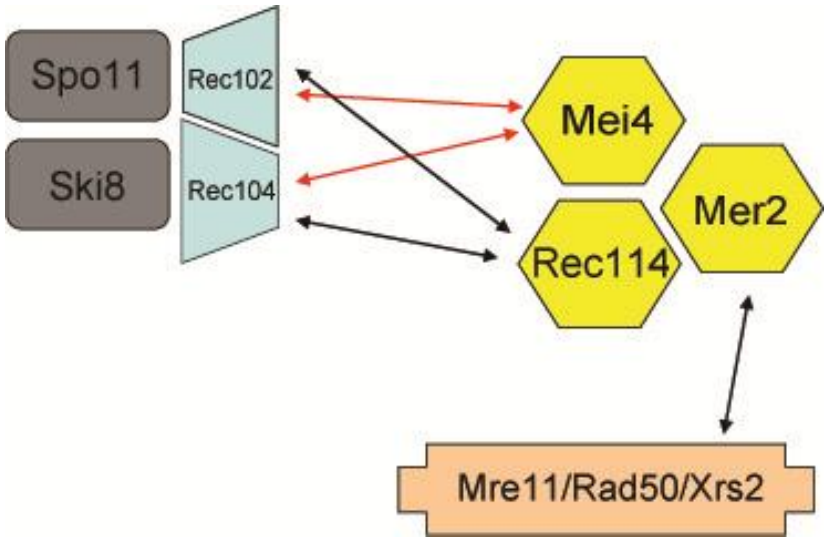


Figure 1.2 A network of meiosis DSB initiation proteins.

Systematic yeast two-hybrid analysis predicted three sub-complexes among all nine meiosis-specific DSB initiation proteins. The Spo11/Ski8/Rec102/Rec104 complex, the Mei4/Mer2/Rec114 complex, and also the Mre11/Rad50/Xrs2 complex. There are extensive genetic and physical interactions between those components within each sub-complex. Vegetative two-hybrid interactions (black arrows) or meiosis-specific two-hybrid (red arrows) also exist between two components from different sub-complexes, as indicated. Figure adapted from (Arora, Kee et al. 2004)

from the cytoplasm to the nucleus in Spo11-dependent manner. Ski8 in turn helps Spo11 bind to Rec102 and Rec104, recruiting them to chromosomes (Arora, Kee et al. 2004). These data supports the theory that Ski8 plays a direct role in DSB formation as a partner of Spo11.

Mutations in *REC102* and *REC104* were first identified in a genetic screen for suppressors of the meiotic lethality in *rad52 spo13* haploids (Malone, Bullard et al. 1991). *REC102* was also identified independently in another screen for sporulation-proficient but meiotic lethal mutants (Bhargava, Engebrecht et al. 1992). *REC102* encodes a 23.2-kDa protein. It is a nuclear-localized protein, but so far Rec102 has no obvious homologs. Rec102 interacts both genetically and physically with Spo11, and it also associates with meiotic chromosomes in a Spo11-dependent manner during early meiotic prophase (Kee and Keeney 2002). Transcription of REC102 is meiosis-specific (Cool and Malone 1992). Null mutants of *rec102* are recombination-defective and produce unviable spores, but the spore unviability can be rescued by a *spo13* mutation (Malone, Bullard et al. 1991; Bhargava, Engebrecht et al. 1992; Cool and Malone 1992).

REC104 encodes a 20.6 KDa protein. It has a few homologs from other Saccharomyces species (*S. paradoxus* and *S. pastorianus*) (Nau, Summers et al. 1997). Transcription of *REC104* is also meiosis-specific, and null mutants of *rec104* show the same phenotypes as those of *rec102* (Galbraith and Malone 1992). Biochemical and genetic evidence indicates that Spo11, Ski8, Rec102 and Rec104 interact with each other during meiotic DSB formation. The association of Rec104 with Spo11 and Ski8 is Rec102-dependent (Jiao, Salem et al. 2003; Kee, Protacio et al. 2004).

Genetic and physical interactions were observed between Mer2, Mei4, and Rec114. Thus, these three proteins form a discrete complex (Li, Hooker et al. 2006;

Maleki, Neale et al. 2007). Based on yeast two-hybrid analysis, the Mer2/Mei4/Rec114 complex interacts with the other factors through Xrs2 and Rec104 (Arora, Kee et al. 2004).

MER2 was isolated in two independent genetic screens, one for suppressors of the intragenic recombination defect in *Δmer1* and *Δmre2* mutants, and the other is for mutations that could rescue the meiotic lethality in *rad52 spo13* haploid strains (Engebrecht, Hirsch et al. 1990; Malone, Bullard et al. 1991; Nakagawa and Ogawa 1997). *mer2* null strains cannot produce viable spores because they cannot generate DSBs, but they do not have any obvious vegetative growth defect (Engebrecht, Hirsch et al. 1990; Malone, Bullard et al. 1991; Cool and Malone 1992; Rockmill, Engebrecht et al. 1995). *MER2* encodes a 35.5kDa protein, which does not have any homologs in other organisms. The *MER2* mRNA contains an 80 nucleotide intron which is only spliced efficiently in meiotic cells. Mer1 stimulates the efficient removal of the non-canonical 5' splice site of Mer2. *MER1* is only transcribed in the meiosis, so Mer2 protein is more abundant in meiotic cells (Engebrecht, Voelkel-Meiman et al. 1991; Nandabalan and Roeder 1995).

MEI4 encodes a 48.1 kDa protein and it is only transcribed during meiosis (Menees, Ross-MacDonald et al. 1992). *MEI4* was first identified in a screen for UV-induced mutants incapable of intragenic meiotic recombination (Menees and Roeder 1989). *mei4* null strains cannot form any meiotic DSBs, and all spores are unviable, but the unviability can be rescued by a *spo13* mutation (Menees and Roeder 1989; Jiao, Bullard et al. 1999).

REC114 was identified together with *REC102* and *REC104* in the screen for suppressors of the meiotic lethality in *rad52 spo13* haploids (Malone, Bullard et al.

1991). It encodes a 49.5kDa protein. The *REC114* transcript contains an intron spliced only during meiosis, but Mer1 is dispensible for splicing of this intron (Malone, Pittman et al. 1997).

Recently, Kumar *et al.* identified orthologs for Mei4 and Rec114 in almost all eukaryotes by using a strategy which identifies small orthologs among increasingly distant species (Kumar, Bourbon et al. 2010). Mei4 was shown to be functionally conserved among species, and *mei4-/-* mice are deficient in meiotic DSB formation as well. They also observed that the discrete foci of Mei4 on the axes of meiotic chromosomes do not overlap with those of DMC1 and RPA. This suggests that Mei4 may act as a structural component which ensures the DSB formation on meiotic chromosomes instead of participating directly in the cleavage of DNA.

THE STRUCTURAL FEATURES OF THE MRX COMPLEX

Similar to its mammalian counterpart Mre11/Rad50/Nbs1 (Dolganov, Maser et al. 1996; Carney, Maser et al. 1998; Paull and Gellert 1998; Trujillo, Yuan et al. 1998), yeast Mre11, Rad50 and Xrs2 proteins make a physical complex (Johzuka and Ogawa 1995; Bressan, Olivares et al. 1998; Usui, Ohta et al. 1998). Rad50 and Mre11 proteins not only are well conserved among all eukaryotic species, but are homologous to SbcC and SbcD proteins from *E. coli*, and have homologs in every organism that has been genetically characterized. The third component is only present in eukaryotes. Nbs1 and Xrs2 are not only similar in size but are also functionally similar to each other.

Yeast Rad50 is a 152-KDa protein with two consensus Walker-type nucleotide-binding motifs (Walker A and Walker B) at the N and C termini, connected by a long central heptad repeat region (Alani, Subbiah et al. 1989). Rad50 belongs to the SMC (Structural Maintenance of Chromosomes) family of proteins due to this general

arrangement. Members of the SMC family are critical for chromosome condensation and segregation in eukaryotes and prokaryotes (Hirano 1999). Yeast Rad50, like other SMC family members, when purified, forms a head-to-tail dimer, in which the Walker A motif at the N terminus of one protomer associates with the Walker B motif at the C terminus of the other protomer. The central region of two protomers form an alpha-helical coiled-coil linked by a flexible hinge in the middle (Raymond and Kleckner 1993) (Figure 1.2).

Mre11 proteins contain sequences in the N-terminus that are conserved in a group of phosphodiesterases (Sharples and Leach 1995) (Figure 1.2). Mre11 alone from human or yeast, or in complex with Rad50 exhibits Mn^{2+} dependent 3' to 5' double-strand exonuclease, and single-strand endonuclease activity (Furuse, Nagase et al. 1998; Paull and Gellert 1998; Trujillo, Yuan et al. 1998; Usui, Ohta et al. 1998; Moreau, Ferguson et al. 1999). Under physiological conditions (5 mM to 10 mM Mg^{2+}), Mre11 has weak 5' endonuclease activity on linear DNA ends (Hopkins and Paull 2008; Nicolette, Lee et al. 2010). Human Rad50 can stimulate the exonuclease activity of Mre11; however, ATP is not required in this stimulatory process.

Nbs1 and Xrs2 both have a fork-head-association (FHA) domain (Durocher, Henckel et al. 1999), and a tandem breast cancer carboxy-terminal (BRCT) domain at the N-terminus (Becker, Meyer et al. 2006), as well as an Mre11-binding motif (MBM) (Shima, Suzuki et al. 2005; Tsukamoto, Mitsuoka et al. 2005) and an ATM/Tel1-binding motif (ABM or TBM) (Nakada, Matsumoto et al. 2003) at the C-terminus (Figure 1.2). The FHA and BRCT domains are phospho-specific protein-protein interaction motifs that are present in many DNA-damage checkpoint proteins (Symington 2002). Xrs2 and Nbs1 are mainly involved in cell cycle checkpoint signaling after DNA damage, through interaction with MRX (N) and Tel1 (ATM)

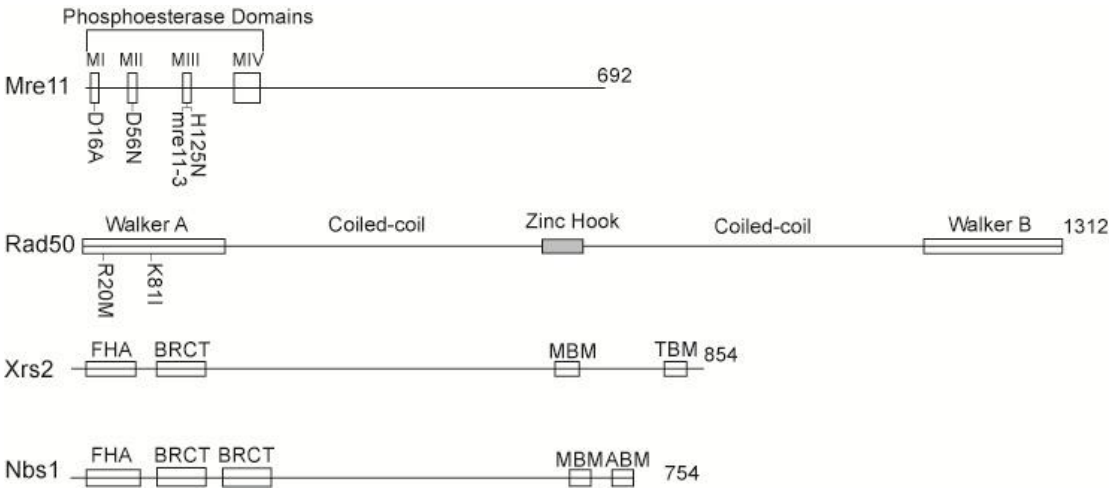


Figure 1.3 Schematic representation of Mre11, Rad50, and Xrs2.

The Mre11 protein contains four phosphodiester motifs (I-IV) at its N-terminus. Mre11-D16A, Mre11-D56N, Mre11-3, Mre11-H125N mutant protein are nuclease-deficient *in vitro* (Furuse, Nagase et al. 1998; Moreau, Ferguson et al. 1999). Rad50 has Walker A and Walker B motifs at its N and C termini, respectively, which are separated by two coiled-coil regions and one zinc-hook domain. Rad50-R20M, and Rad50-K81I are *rad50S* mutants (Alani, Padmore et al. 1990; Cao, Alani et al. 1990; Sun, Treco et al. 1991). Both Xrs2 and Nbs1 contain FHA and BRCT domains at their N-termini. Xrs2 has an Mre11-binding motif (MBM) and a Tel1-binding-motif (TBM) at its C-termini, whereas Nbs1 has one MBM and one ATM-binding-motif (ABM). (Figure adapted from (Krogh and Symington 2004))

(D'Amours and Jackson 2001; You, Chahwan et al. 2005). Xrs2 and Nbs1 also assist the nuclease role of Mre11 in DNA end processing and DNA repair (Paull and Gellert 1999; Trujillo, Roh et al. 2003). Recent genetic, biochemical, and structural evidence indicated that Nbs1 can tether Ctp1 (the functional homolog of Sae2 in fission yeast) and MR complex flexibly through its FHA domain, thus it recruits Ctp1 to the immediate vicinity of DSBs. (Williams, Dodson et al. 2009)

THE ROLE OF MRX IN DNA REPAIR

In many organisms, including *S. pombe* (Tavassoli, Shayeghi et al. 1995), *C. elegans* (Chin and Villeneuve 2001), *C. cinereus* (Gerecke and Zolan 2000), N. crassa (Watanabe, Sakuraba et al. 1997), *D. melanogaster* (Ciapponi, Cenci et al. 2004), *A. thaliana* (Bundock and Hooykaas 2002), and *S. cerevisiae*, null mutants of *Mre11* or *Rad50* exhibit hypersensitivity to DNA damaging agents. The MR complex has also been demonstrated to be involved in DSB repair through both homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways, as well as in cell cycle responses to DNA damage and telomere maintenance, as reviewed in (Haber 1998; Paques and Haber 1999; Petrini 1999). It appears that the role of this complex in somatic cells is well conserved. The loss of any components in MRN complex even causes lethality in vertebrate cells (Yamaguchi-Iwai, Sonoda et al. 1999), and in mice, all three genes of the MRX complex are indispensible for early embryonic development (Xiao and Weaver 1997; Luo, Yao et al. 1999; Yamaguchi-Iwai, Sonoda et al. 1999).

The MRX complex is required for meiotic recombination in sexually reproducing organisms, and in some organisms (including *S. cerevisiae*) it is also required for meiotic DSB formation as well (Malone and Esposito 1981; Alani, Padmore et al. 1990; Cao, Alani et al. 1990; Ivanov, Korolev et al. 1992; Ajimura, Leem et al. 1993).

SEPARATION OF FUNCTION MUTANTS OF THE MRX COMPLEX

In *S. cerevisiae*, some non-null mutants of the MRX complex cause covalent Spo11-DSB adducts to accumulate during meiotic prophase I and block sporulation (Alani, Padmore et al. 1990; Cao, Alani et al. 1990; Nairz and Klein 1997). These mutants are named *mre11S* and *rad50S* mutants. "S" stands for "Separation-of-function" alleles that are defective in meiosis but show nearly wild-type resistance to DNA damage in vegetative cells. The phenotypes of the *rad50S* and *mre11S* mutants suggested that MRX is involved in the removal of Spo11 from DSBs during prophase in meiosis I. In addition, *mre11* nuclease-deficient mutants (*mre11-58S* and *mre11- H125N*) have the same phenotype as *rad50S* strains (Tsubouchi and Ogawa 1998; Symington, Kang et al. 2000). This result suggests that Mre11 nuclease activity is required for protein-DNA adduct removal and 3' overhang formation.

IDENTIFICATION OF SAE2

In addition to Mre11, another candidate for such a meiotic DSB end processing activity is Sae2. *SAE2/COM1* (for Sporulation in the Absence of Spo Eleven, or Completion of meiotic recombination) was identified in two independent screens for mutations that conferred SPO11-dependent sporulation defects. *Sae2/COM1* null mutants also accumulate Spo11-DNA covalent complexes during meiosis and show the same sporulation phenotype as *rad50S* mutants (McKee and Kleckner 1997; Prinz, Amon et al. 1997). This suggests that Sae2 may cooperate with MRX during meiotic DSB repair. However, there are no obvious motifs of known biochemical function present in the 40kDa Sae2 protein sequence. Sae2 shares very limited homology at its C-terminus with its functional counterpart CtIP/Ctp1 in *H. Sapiens*, *C. elegans*, *D. melanogaster*, *S. pombe* (Huertas, Cortes-Ledesma et al. 2008). Two signature motifs are within this

region: the stretches of residues of RHR and FPSTQ are shared by many eukaryote species. The C terminal part of Sae2 also includes several conserved Serine/Threonine residues, which are potential phosphorylation sites for ATM/ATR or CDK (Figure 1.4, Figure 4.1, and Figure 4.3).

ROLES OF THE MRX COMPLEX AND SAE2 IN MITOTIC DSB REPAIR

In addition to the cooperative roles played by MRX and Sae2 in meiotic DSB resection, Rattray *et al.* found that Sae2 also participates in DNA recombination in vegetative cells. Large palindromic duplication products accumulated in *Δsae2*, *rad50S* and *mre11* nuclease-deficient strains, due to improper processing of hairpin fold-back structures created by a DSB within an inverted repeat (Rattray, McGill et al. 2001; Rattray, Shafer et al. 2005).

Lobachev *et al.* also described the intersection between the functions of Sae2 and MRX in the processing of hairpin-capped ends in *S. cerevisiae* using a different genetic assay in vegetatively growing cells (Lobachev, Gordenin et al. 2002). They inserted closely-spaced inverted repeats into the *LYS2* gene on chromosome II while a 5' truncated *lys2* allele was present on chromosome III. Spontaneous DSBs were observed at the site of the inverted repeats, indicating that the repeats likely extruded into cruciform structures *in vivo*, and these structures were highly susceptible to breakage. The frequency of homologous recombination between the two *lys2* alleles increased over 1000-fold, and was dependent on the presence of the inverted repeats. In wild-type cells, such homologous recombination results in an intact *LYS2* gene. *Δsae2*, *rad50S*, *Δmrx* and Mre11 nuclease-deficient strains failed to process the cruciform structures. This instead led to the accumulation of chromosomes with hairpin-capped ends at the sites of the inverted repeats, and large chromosome amplifications of the entire chromosome arm

next to the repeats. Taken together, these data suggest that Sae2 and the MRX complex are critical for the processing of DNA hairpin structures in *S. cerevisiae*, and that Mre11 nuclease activity plays a unique role in this process as well.

SAE2 ENDONUCLEASE ACTIVITY IN VITRO

From the Paull lab, in collaboration with A. Rattray, Lengsfeld et al. showed that Sae2 exhibits endonuclease activity *in vitro*, with a preference for single-stranded DNA, and single-stranded/double-stranded DNA junctions *in vitro*. They confirmed that Sae2 had a preference for hairpin substrates and that MRX facilitated Sae2 endonuclease activity on single-stranded DNA adjacent to a hairpin. MRX also stimulated Sae2 endonuclease cutting through its 3' to 5' exonuclease activity (Lengsfeld, Rattray et al. 2007). Their results showed that the conserved C-terminus of the protein contributed to cooperative hairpin removal with MRX, but was not essential for endonuclease activity. Other point mutants (G270D) were deficient in endonuclease activity, although this is likely due to their deficiency in DNA binding. Thus, the catalytic residues of Sae2 are not yet identified and have become one of the questions I tried to address in my investigation of the Sae2 protein and its roles in DNA repair.

TEL1/MEC1 PHOSPHORYLATION OF SAE2

Sae2 is a phosphorylation target of the Mec1/Tel1-dependent checkpoint in response to DNA damage. Mec1 and Tel1 are the major DNA damage signaling kinases in yeast, analogous to ATR/ATM in mammalian cells. This family of kinase is specific for the target sequence (S/T)Q. When all five putative (S/T)Q phosphorylation sites in Sae2, S73, T90, S249, T279, and S289 were mutated to alanine, Sae2 phosphorylation was blocked *in vivo*, and strains expressing this Sae2 (5A) mutant show increased

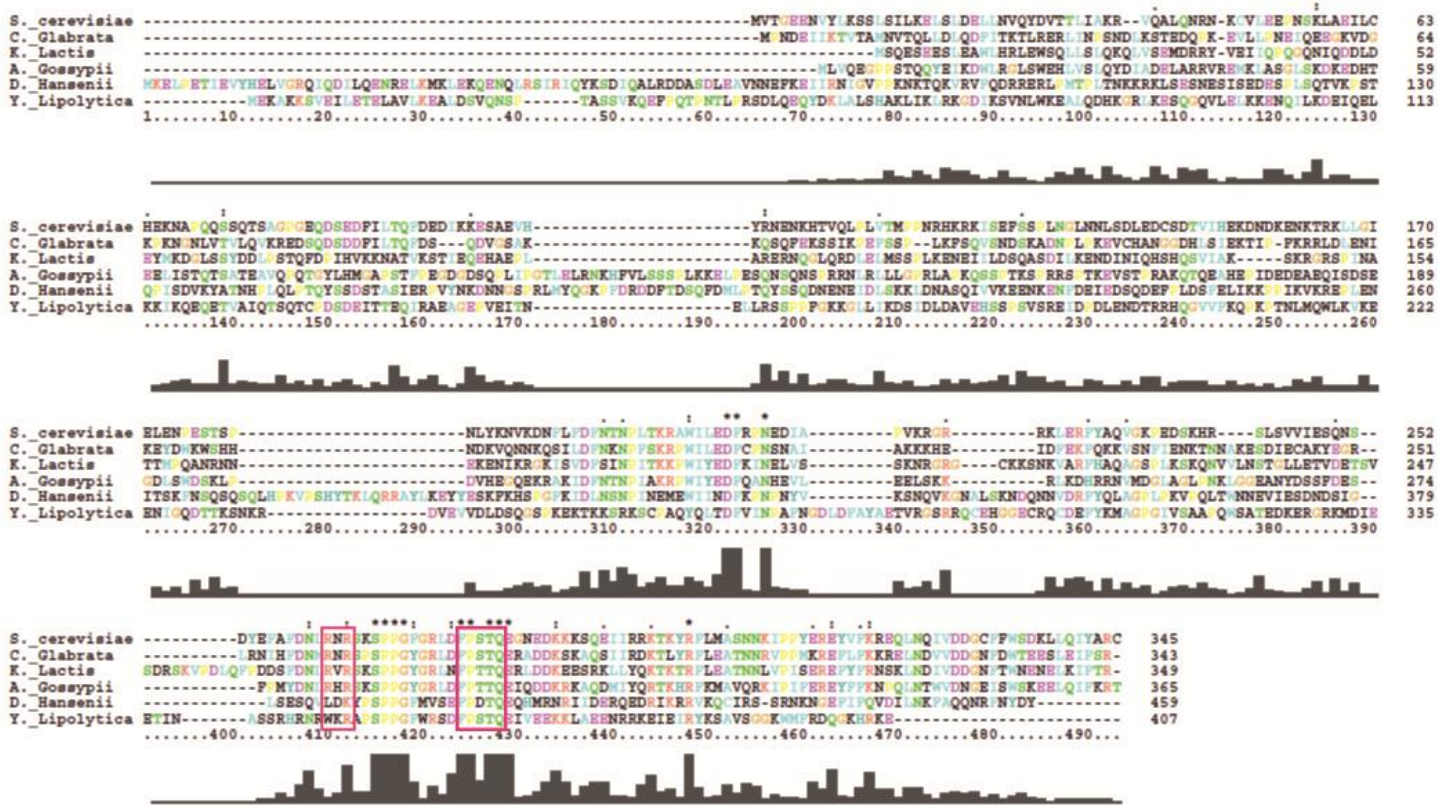


Figure 1.4 Alignment of homologs of Sae2 from fungi.

A sequence alignment of Sae2 from *S. cerevisiae*, *A. Gossypii*, *C. Glabrata*, *D. Hansenii*, *K. Lactis*, *Y. Lipolytica* was performed in Clustal X 2.1. "*" indicates a fully conserved residue. The RHR and FPSTQ motifs are highlighted in red rectangles.

sensitivity to MMS in comparison to strains expressing wild-type Sae2 (Baroni, Viscardi et al. 2004). *In vitro*, the Sae2 (5A) mutant showed an intermediate level of endonuclease activity (Lengsfeld, Rattray et al. 2007), and it lost its nuclease activity on hairpins that is normally seen cooperatively with MRX. However, the activity of Sae2 (5A) *in vitro* may not exactly reflect its activity in yeast, because recombinant Sae2 purified from *E. coli* cells does not have any phosphorylation modifications yet it is catalytically active. Thus, the 5A mutations must be altering the characteristics of the protein in a way that is independent of phosphorylation. It is possible that Sae2 phosphorylation by Tel1/Mec1 may regulate its dynamics with other proteins, like MRX, upon DNA damage, instead of directly regulating its endonuclease activity.

Sae2 also negatively regulates the Mec1/Tel1-dependent DNA damage checkpoint signaling (Gardner, Putnam et al. 1999). In wild-type cells, Rad53 is phosphorylated by Mec1/Tel1 in response to DNA damage, which is a hallmark of checkpoint arrest. Rad53 will be later dephosphorylated during the recovery from the cell cycle arrest. However, after introduction of an irreparable DSB, cells lacking Sae2 or expressing the Sae2 (5A) mutant failed to turn off Rad53 phosphorylation. In contrast, overexpression of Sae2 prevents Rad53 phosphorylation after DNA damage and impairs MRX foci formation in the presence of unrepaired DSBs. This indicates that Sae2 affects the duration of Mec1/Tel1 signaling through Rad53, possibly by modulating the association of MRX to DSBs (Usui, Ogawa et al. 2001; Baroni, Viscardi et al. 2004; Clerici, Mantiero et al. 2005; Usui, Petrini et al. 2006).

CDK PHOSPHORYLATION OF SAE2

Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways used to repair DNA DSBs. HR is a more accurate DSB repair

mechanism because it occurs exclusively in the S and G_2 phase of the cell cycle, when DNA has been replicated and a sister chromatid is available to serve as a repair template. Although NHEJ occurs through the entire cell cycle, it does have more importance in G1 phase. NHEJ usually rejoins two DNA ends directly with no or little homology.

CDK regulates the choice between HR and NHEJ, primarily through its control of DSB resection (Huertas, Cortes-Ledesma et al. 2008; Kosugi, Hasebe et al. 2009). DSB resection is essential for HR, but not NHEJ. Sae2 is one of the important targets of CDK to regulate homologous recombination. Sae2 is regulated by CDK phosphorylation at Ser267 (Huertas, Cortes-Ledesma et al. 2008), which is located in the evolutionary conserved C-terminus motif of Sae2. Cells expressing a non-phosphorylatable S267A mutant have phenotypes comparable to a *sae2* null mutant, including hypersensitivity to DNA-damaging regents, loss of meiotic recombination, impaired DNA end-processing, and reduced hairpin-induced homologous recombination. In contrast, an S267E mutant mimicking constitutively Ser267 phosphorylation retains wild-type phenotypes.

SAE2 ACETYLATION

The Foiani laboratory recently demonstrated that, in addition to phosphorylation, Sae2 is also acetylated (Robert, Vanoli et al. 2011). Acetylation was shown to regulate the stability of Sae2 protein in yeast. After the inhibition of deacetylases (HDACs), acetylated Sae2 was degraded through an ATG-1 mediated autophagy pathway. In my study, I illustrated that cells accumulate about 3-fold higher level of Sae2 in *atg1* yeast that are deficient in the autophagy pathway. The regulatory function of this modification and the sites of acetylation are currently under active investigation.

THE ROLE OF CTIP/CTP1 IN RESECTION

The functional ortholog of Sae2 in other eukaryotes, CtIP (mammalian cells, chicken cells, Xenopus)/Ctp1 (fission yeast)/Com1 (worms, plant) shares a limited conservation with Sae2 at the C-terminus of the protein, but functionally, they play a remarkably similar and indispensable role in DSB end processing (Nakamura, Kogame et al. ; Chen, Liu et al. 2005; Penkner, Portik-Dobos et al. 2007; Uanschou, Siwiec et al. 2007).

In fission yeast, Ctp1 was demonstrated to be required for efficient resection as well in S and G_2 phases of the cell cycle. However, unlike Sae2 in budding yeast, Ctp1 was regulated at the level of transcription, so that it was more available during S and G_2. (Limbo, Chahwan et al. 2007)

In human U2OS cells and chicken DT-40 cells, CtIP has been shown to be important for resection in S and G_2 phases in cell cycles, and it was also governed by CDK to regulate DSB resection in a similar way as that of Sae2 (Huertas and Jackson 2009; You, Shi et al. 2009). The residue analogous to S267 in Sae2 is T847 in human CtIP. Cells expressing non-phosphorylatable CtIP (T847A) were more sensitive to camptothecin treatment than cells expressing wild-type CtIP. In contrast, cells expressing CtIP (T847E) were more resistant to camptothecin. Further analysis revealed that cells expressing CtIP (T847D) promoted resection at DSB ends in response to DNA damage equally as well as cells expressing wild-type CtIP did. In human CtIP, CDK has another target site S327, through which CtIP interacts with BRCA1. This interaction also plays an important role in checkpoint responses to DNA damage and DSB resection (Chen, Nievera et al. 2008).

Recently, human CtIP has been characterized as a target of acetylation/deacetyleation as well. CtIP was found to be constitutively acetylated in undamaged cells, and after DNA damage, it was deacetylated by SIRT6 (Kaidi, Weinert et al.). This apparent decrease in acetylation level in CtIP has been shown to promote its DNA end processing ability. When cells were treated with nicotinamide (an inhibitor of the NAD^+ dependent sirtuin family of KDACs (SIRT1 to SIRT7)) or siRNA against *SIRT6*, cells accumulated acetylated CtIP and exhibited a significantly lower level of RPA phosphorylation at Ser4 and Ser8, which is a marker of resected DSB ends.

REDUNDANT DNA END PROCESSING PATHWAYS IN MITOTIC CELLS

In meiotic budding yeast cells, there is a strict requirement for Mre11 nuclease activity and the Sae2 protein. However, in mitotic cells, resection of DSBs is only delayed in the absence of Mre11 or Sae2, and is nearly unaffected when *Mre11* was mutated at the nuclease active site. This indicated that there must be other redundant pathways responsible for DNA ends processing in vegetative growing yeast cells.

Several groups demonstrated that DSB resection *in vivo* occurs in two stages: an initial short-range resection (50 to 300 nt), followed by long-range resection of several thousand nucleotides or more. MRX and Sae2 together were shown to be important for short-range resection, and also for the efficiency of long-range resection (Mimitou and Symington 2008; Zhu, Chung et al. 2008; Nicolette, Lee et al. 2010; Niu, Chung et al. 2010). Long-range resection depends on two redundant pathways, the 5' to 3' endonuclease and exonuclease Exo1, and the 5' flap endonuclease Dna2, which acts together with helicase Sgs1/RmiI/Top3. Abrogation of either one of these two pathways had little effect on DNA end processing. However, deletion of both pathways blocked

long-range resection, and left the broken DNA ends only to be processed up to 50 to 300 nt from the 5' end (Mimitou and Symington 2008; Zhu, Chung et al. 2008).

Using *in vitro* analysis of purified proteins, Nicolette *et al.* clarified that MRX and Sae2 could stimulate Exo1 activity on DNA ends using recombinant proteins. The primary effect of MRX and Sae2 was in promoting Exo1 binding to DNA ends, which did not require the nuclease activity of either complex. There was also a secondary stimulatory effect of MRX and Sae2 that was mediated by their ability to remove the 5' strands and create short 3' overhangs (Nicolette, Lee et al. 2010). Niu *et al.* also reconstituted the other DNA end-resection pathway comprising MRX, Sgs1-Top3-Rmi1, Dna2, and RPA. The nuclease activity of Dna2 was found to be essential for this reaction while that of MRX is not (Niu, Chung et al. 2010). Notably, RPA played different roles in these two redundant pathways. RPA increased the efficiency of resection catalyzed by Dna2 and the Sgs1 complex because RPA stimulated DNA unwinding by Sgs1, specifically bound with the 3′ single strands, and protected them from degradation by Dna2. However, RPA inhibited the activity of Exo1 *in vitro*, although MRX and Sae2 could partially restore Exo1 activity in the presence of RPA (Nicolette, Lee et al. 2010; Niu, Chung et al. 2010).

INTERPLAY BETWEEN KU AND MRX/SAE2

In budding yeast, the MRX complex is indispensible for both HR and NHEJ, whereas in other eukaryotes (fission yeast, mammals), MRX is primarily required for HR and plays accessory roles in NHEJ. After DSBs form, MRX is rapidly recruited to DSB sites and initiates the signaling of checkpoint activation through Tel1 kinase, tethers the broken DNA ends together by the long coiled-coil structure of Rad50, and regulates the initiation of 5' to 3' end processing. NHEJ in budding yeast also requires MRX

(Dudasova, Dudas et al. 2004; Daley, Vander Laan et al. 2005), and MRX has been shown to promote ligase IV binding to DSB ends (Wu, Topper et al. 2008).

Ku plays an essential role in NHEJ. In yeast, Ku is a heterodimer of Hdf1 (yeast Ku70) and Hdf2 (yeast Ku80). They form a ring-shaped molecule which binds DNA and protects DNA ends efficiently by threading through its hole in the middle of the ring (Walker, Corpina et al. 2001). Once bound, Ku recruits downstream NHEJ factors, Lif1, Nej1 and Dnl4 (Daley, Vander Laan et al. 2005).

The interplay between MRX and Ku defines the choice between HR or NHEJ pathways in cells. Deletion of Ku was found to increase the initiation of resection significantly at the DSB ends as well as at telomeres (Lee, Moore et al. 1998; Maringele and Lydall 2002; Clerici, Mantiero et al. 2008). In *Δ mre11* or *Δ rad50* strains, deletion of Ku partially restores their IR and MMS resistance (Bressan, Baxter et al. 1999; Wasko, Holland et al. 2009). Genetic analysis revealed that such suppression was dependent on Exo1. In addition, deletion of Ku almost fully rescues the IR hypersensitivity of *Δsae2* mutants (Mimitou and Symington 2010). In addition, *Δ mre11* or *Δ rad50* strains were shown to accumulate significantly more Ku at the DSBs ((Mimitou and Symington 2010; Shim, Chung et al. 2010). These results suggest that MRX and Ku compete at DSB ends and that during S and G_2 phase, MRX together with Sae2 promotes Exo1 exonuclease activity at DSB ends by alleviating the inhibitory effect of Ku. *In vitro* analysis using recombinant protein complexes also favors this hypothesis, in which inhibitory effect on Exo1 by Ku can be partially reversed in the presence of MRX and Sae2 ((Mimitou and Symington 2010; Shim, Chung et al. 2010).

HYPOTHESIS AND GOALS

Meiotic DSB formation and processing

Although successful DSB formation has been shown to require ten proteins in yeast, the specific role of each of them is poorly understood. To date, we have known little about whether these accessory proteins directly participate in DSB formation during meiosis or simply regulate the accessibility of Spo11 and other proteins. Spo11 has never been shown directly to cleave DNA. Spo11 is difficult to express in recombinant form and Spo11 enzymatic activity has never been demonstrated *in vitro*. I hypothesize that Spo11 initiates meiosis DSB formation by first forming a covalent 5'-phosphodiester linkage with the newly created 5' DNA end, with the assistance of its close interaction partners, Ski8, Rec102, and Rec104. The role of MRX and Sae2 is likely to remove Spo11-oligonucletotide adducts from DSB sites, and facilitate DSB 5' strand processing. Understanding the mechanisms for meiotic DSB formation and processing in budding yeast will provide useful insights into the similar machinery in higher organisms, including humans.

Sae2 structure and function

Sae2 exhibits endonuclease activity in vitro, but it contains no obvious nuclease domain. Sequence alignment of Sae2 in several species related to *S. cerevisiae* only reveals a short stretch of conserved residues at the C terminus of Sae2. One of the problems I tried to solve in my study is to characterize the active sites of Sae2 responsible for its nuclease activity. Sae2 is also a target of several kinds of post-translational modification, in response to DNA damage or during cell cycle progression. Previous studies suggest that such modification as phosphorylation by CDK, and phosphorylation by Tel1/Mec1 is critical for the function of Sae2 at the DSB sites. It remains a challenge

to characterize what effects these modifications have on Sae2 function, or on its functional partner MRX. My goal is to clarify the function of phosphorylated Sae2 in DNA repair by using purified recombinant Sae2, and to characterize the non-canonical mechanism of the effect of this endonuclease Sae2 on DSB repair.

CHAPTER 2: MATERIALS AND METHODS

PLASMID CONSTRUCTION

Protein Expression Constructs for E. coli

The wild-type Sae2 expression construct, pExp566.gck, was a gift from A. Rattray. The *SAE2* gene in this construct is under the control of a T7 promoter to express Sae2 with a 6X Histidine tag and a Maltose Binding Protein (MBP) tag fused at the N-terminus. pTP1462 (*sae2 (L25P)*), pTP2111 (*sae2 (S84A/E85G/D86G/F87L)*), pTP1221 (*sae2 (D193A)*), pTP950 (*sae2 (N197I)*), pTP1103 (*sae2 (P198L)*), pTP1745 (*sae2 (W204L)*), pTP2052 (*sae2 (I205G)*), pTP1818 (*sae2 (E207A/D208Q)*), pTP1386 (*sae2 (N212G)*), pTP1382 (*sae2 (E226A)*), pTP1871 (*sae2 (D275A)*), pTP951 (*sae2 (E281A)*) were constructed by Quikchange mutagenesis using primers TP2123/TP2124, TP2950/TP2951, TP1306/TP1307, TP1274/TP1275, TP1690/1691, TP2239/TP2240, TP2241/TP2242, and TP2600/TP2601, TP2031/TP2032, TP2033/2034, TP1308/TP1309, and TP1276/1277 respectively. CDK phosphorylation mimic and phosphorylation deficient Sae2 mutant expression plasmids, pTP1176 (*sae2 (S267A)*), and pTP1172 (*sae2 (S267E)*) were constructed by Quikchange mutagenesis using primers TP1809/TP1810, and TP1811/TP1812 respectively. The expression construct pTP1095 (*sae2 (S73D/T90D/S249D/T279D/S289D, or 5D)*) was described previously (Lengsfeld, Rattray et al. 2007). pTP1173 (*sae2 (5D/S267E)*) was constructed by Quikchange mutagenesis using pTP1095 and primers TP1811/TP1812. Sae2 (CD, central domain, a.a. 175-268) expression construct pTP1282 was constructed by PCR the coding sequence for a.a. 175-268 using primer TP1874/TP1875 from pEXP566.gck first. The PCR fragment was introduced after the MBP coding sequence in pMalC2X at *BamH*I and *Hind*III sites.

Table 2.1 Primers used in this study.

Primer Name	Sequence
TP2950	5’- GCCAGGTGAGCAAGATGCTGGAGGTTTAATCCTTACTCAGTTTG -3’
TP2951	5’- CAAACTGAGTAAGGATTAAACCTCCAGCATCTTGCTCACCTGGC -3’
TP2123	5’- AGCTCAGTCTCGATGAACCACTAAATGTGCAGTATGA -3’
TP2124	5’- TCATACTGCACATTTAGTGGTTCATCGAGACTGAGCT -3’
TP2600	5’- AGCCTGGATTCTCGCACAGTTTAGACCAAATGAAGATA -3’
TP2601	5’- TATCTTCATTTGGTCTAAACTGTGCGAGAATCCAGGCT -3’
TP1800	5’-TCTAGACCGCGGCTTTCTCATCTTC-3’
TP1801	5’-CATATGACAAACACCTAATGGCTTAC-3’
TP790	5’- GGATCCGCGGCCGCATGGCTTTGGAGGGATTGCGG -3’
TP791	5’- CTGCAGACTAGTTCATTTGTATTCAAAAATTCTGGC -3’
TP1409	5’- GGATCCATGAAGCTACTGTCTTC -3’
TP1411	5’- AGATCTCGATACAGTCAACTGTCTTTG -3’
TP1478	5’- CAGTGAGAGATATCTTCTTCTCCAACGTGGAATTG -3’
TP1479	5’- CAATTCCACGTTGGAGAAGAAGATATCTCTCACTG -3’
TP1386	5’- AAGCTTATGTCCAAAGTGTTTATTGCC 3’
TP1387	5’- CTCGAGTTTACCGCCAGCTTCTCTAAAC -3’
TP910	5’- CTCGAGCTTATTGTACGTTACAGCCGGTAAGG -3’
TP911	5’- GTCGACATGAACGATCGATTAGTTACGGAAGAGCAAGAG -3’
TP1840	5’- GTCGACATGGCAAGAGATATCACATTTTTGA -3’
TP1895	5’- TAATCGATCGTTCATTTTAAAATTCAGTTTAATTTTCAA

CATTCCAGGTGTAGCAGT -3’

TP1375 5’- GGATCCATGTCCATCGAGGAGGAAGATAC -3’

TP1496 5’- ACTAGTTCAGGGACTACTAAACTGAAATATG -3’

TP915 5’- GTCGACATGGTCGCTAGAGGTAGAACAGACGAG -3’

TP914 5’- CTCGAGCAGCTCAGATTCCAGAGTGTCGGGTC -3’

TP1377 5’- CAAGATGCTGCTACGAACGGAAACGTGAAAACC -3’

TP1378 5’- GGTTTTCACGTTTCCGTTCGTAGCAGCATCTTG -3’

TP1936 5’- GATTGAATTAAAGCAAAACAGATTATCCAGTCAACTTCCG -3’

TP1937 5’- CGGAAGTTGACTGGATAATCTGTTTTGCTTTAATTCAATC -3’

TP909 5’- GGATCCATGTACGAGTACTGCTCAGTTGTAATAAAG -3’

TP1526 5’- ACTAGTTCACTTTTCGAACATTTTATTGAGAACCG -3’

TP1934 5’- ACCGTTTCAACCTTATTAACCCATTTCATGAATTCTTCGT -3’

TP1935 5’- ACGAAGAATTCATGAAATGGGTTAATAAGGTTGAAACGGT -3’

TP1809 5’-CTTGAGGAATAGATCAAAAGCCCCCCCAGGTTTTGG-3’

TP1810 5’-CCAAAACCTGGGGGGGCTTTTGATCTATTCCTCAAG-3’

TP2516 5’- TGCTATGTGGCGCGGTATTAT-3’

TP2517 5’- CTGTCATGCCATCCGTAAGATG-3’

TP2518 5’- 6FAM-CAAGAGCAACTCGGTCGCCGCATA-TAMRA-3’

TP1524 5’- CCCGCTCGGAGCACTCTCCTCGGTACTTC -3’

TP1525 5’- GAAGTACCGAGGAGAGTGCTCCGAGCGGG -3’

TP74 5’- CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCA -3’

TP1152 5’-
CATCTGGCCTGTCTTACACAGTGCTACAGACTGGAGTCCTCATCAGACTG-3’

TP124 5’-

	CATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG-3’
TP2031	5’-AAGACTTTAGACCAGGTGAAGATATCGCCCC-3’
TP2032	5’-GGGGCGATATCTTCACCTGGTCTAAAGTCTT-3’
TP1306	5’-GGATAATTTCCTGTTTGCCTTCAATACGAACCCTTTAAC-3’
TP1307	5’-GTTAAAGGGTTCGTATTGAAGGCAAACAGGAAATTATCC-3’
TP2033	5’-GAAGAAGAAAATTGGCGCGATTTTATGCCCA-3’
TP2034	5’-TGGGCATAAAATCGCGCCAATTTTCTTCTTC-3’
TP2239	5’-CCTTTAACAAAGCGAGCCTTGATTCTCGAAGACTTTAG-3’
TP2240	5’-CTAAAGTCTTCGAGAATCAAGGCTCGCTTTGTTAAAGG-3’
TP2241	5’-CTTTAACAAAGCGAGCCTGGGGTCTCGAAGACTTTAGACC-3’
TP2242	5’-GGTCTAAAGTCTTCGAGACCCCAGGCTCGCTTTGTTAAAG-3’
TP1274	5’-GTTTGACTTCAATACGATCCCTTTAACAAAGCG-3’
TP1275	5’-CGCTTTGTTAAAGGGATCGTATTGAAGTCAAAC-3’
TP1690	5’-GGCTCGCTTTGTTAAAAGGTTCGTATTGAAGTC-3’
TP1691	5’-GTATTCAAAAGAGAACAAATAAATCAAATTGTTGACG-3’
TP1276	5’-TCCCTCCACTCAGGCAGGGAACGAGGACAA-3’
TP1277	5’-TTGTCCTCGTTCCCTGCCTGAGTGGAGGGA-3’
TP1308	5’-GGTTTTGGAAGACTGGCTTTTCCCTCCACTCAGG-3’
TP1309	5’-CCTGAGTGGAGGGAAAAGCCAGTCTTCCAAAACC-3’
TP1811	5’-CTTGAGGAATAGATCAAAAGAACCCCCAGGTTTTGGAAG-3’
TP1812	5’-CTTCCAAAACCTGGGGGTTCTTTTGATCTATTCCTCAAG-3’
TP2553	5’- AAGGCCTTGCCTATCGGGACCAGATCGACAAGCGTACAGGTAT ACCTGTACGCTTGTC -3’

TP2493	5’- GAGATGGCGCCCAACAGT-3’
TP2495	5’- 6FAM-ACGCCGAAACAAGCGCTCATGAG-TAMRA-3’
TP2494	5’-AAGATCGGGCTCGCCACT-3’
TP1874	5’- GGATCCCCTGAATCTACATCGCCAA-3’
TP1875	5’- CTGCAGGCGGCCGCTTAGGGGGATTTTGATCTATTCC -3’

The MBP-Sae2 (CD) coding sequence was cloned into pEXP566.gck using *Bgl*II/*Not*I sites to yield pTP1282.

Yeast Complementation Constructs

pTP1350 contains the Sae2 coding sequence with a 6X Histidine and MBP tag fused at the N-terminal end, under the control of the native *SAE2* promoter from *S. cerevisiae*. The *SAE2* promoter region was PCR amplified using primers TP1800 and TP1801 from wild-type *S. cerevisiae* strain W303α, with 5' *Xba*I and 3' *Nde*I restriction sites, respectively. The PCR fragment was introduced before HIS-MBP-Sae2 coding sequence in pEXP566.gck at *Xba*I and *Nde*I sites. The entire *SAE2* promoter region followed by HIS-MBP-Sae2 coding sequence was cloned into pRS313 using *Sac*II/*Xba*I sites to yield pTP1350. Mutants of the *SAE2* gene in pTP1350 were constructed by using the Quikchange mutagenesis kit (Stratagene), similar to pTP2111, and pTP2140. Quikchange mutagenesis using primers TP2123/TP2124, TP2950/TP2951, TP1306/TP1307, TP2239/TP2240, TP2241/TP2242, TP2031/TP2032 and TP2033/TP2034 yielded *sae2 (L25P)* in pTP1477, *sae2 (S84A/E85G/D86G/F87L)* in pTP2110, *sae2 (D193A)* in pTP1394, *sae2 (W204L)* in pTP1537, *sae2 (I205G)* in pTP1538, *sae2 (N212G)* in pTP1474, and *sae2 (E226A)* in pTP1473, respectively.

pLEU2promFlag-SAE2 (gift from J. Petrini) contained *SAE2* with a 2XFlag tag fused at the N-terminal end, under the control of the native *SAE2* promoter from *S. cerevisiae* in pRS425. Mutants of the *SAE2* gene in pLEU2promFlag-SAE2 were constructed by using the Quikchange mutagenesis kit (Stratagene), similar to pTP2111, and pTP2140. Quikchange mutagenesis using primers TP2123/TP2124, TP2950/TP2951, TP1306/13107, TP2239/TP2240 and TP2241/TP2242 yielded *sae2 (L25P)* in pTP1517,

sae2 (S84A/E85G/D86G/F87L) in pTP2109, *sae2 (D193A)* in pTP2009, *sae2 (W204L)* in pTP2011*, and sae2 (I205G)* in pTP2008, respectively.

Baculovirus Expression Constructs

Expression constructs were made for the expression of MBP-Spo11 and Gal4-MBP-Spo11 using baculovirus in Sf21 insect cells. The *SPO11* gene was PCR amplified from the wild-type *S. cerevisiae* strain W303α using primers TP790 and TP791 with 5' *Not*I and 3' *Spe*I sites, respectively. The *SPO11* PCR product was cloned into pCR-BLUNT-II-TOPO yielding pTP431. The MBP tag, together with *SPO11* PCR product from pTP431, was introduced into pFastbac1 at *BamH*I/*Not*I and *Not*I/*Spe*I sites, respectively, yielding pTP923. The Gal4-Binding domain sequence was PCR amplified from pAS2-1 with TP1409, and TP1411, and introduced into pTP923 at *BamH*I and *Bgl*II sites to yield pTP933. pTP933 was made into a bacmid as previous described (Bhaskara, Dupre et al. 2007) to make pTP941. The *spo11 (Y135F)* expression construct, pTP962, was modified by Quikchange mutagenesis using primers TP1478 and TP1479. pTP962 was made into a bacmid as described above to make pTP967.

SKI8 was PCR amplified from pAJ706 (a gift from Dr. Arlen Johnson) using primers TP1386 and TP1387 with 5' *Hind*III and 3' *Xho*I sites, respectively. The *SKI8* PCR product was cloned into the pFastbac1 vector with a C-terminal 6X Histidine tag to yield pTP911. pTP911 was made into a bacmid pTP921.

Exon 1 and exon 2 of *REC102* were PCR amplified separately from the wild-type *S. cerevisiae* strain W303α with primers TP910/TP911, or TP1840/TP1895, respectively. The PCR products were then introduced into the pFastbac1 vector using *Sal*I/*Cla*I, and *Sal*I/*Xho*I restriction sites, to yield Rec102 with a C-terminal 6X Histidine tag coding sequence in pTP1333. pTP1333 was made into a bacmid pTP1344.

REC104 was PCR amplified from wild-type *S. cerevisiae* strain W303α using primers TP1375/TP1496 with 5'*BamH*I and 3' *Spe*I. The *REC104* PCR product was introduced into the pFastbac1 vector with an N-terminal Flag-tag to yield pTP976. pTP976 was made into a bacmid pTP988.

MER2 was PCR amplified from the wild-type *S. cerevisiae* strain W303α using primers TP915/TP914 with 5' *Sal*I and 3' *Xho*I sites, respectively. The intron of Mer2 was removed using overlapping PCR with primers TP1377 and TP1378. Mer2 coding sequence PCR product was introduced into the pFastbac1 vector with a C-terminal 6X hisitidine tag using restriction sites *Sal*I and *Xho*I to yield pTP990. pTP990 was made into bacmid pTP1000.

MEI4 was PCR amplified from the wild-type *S. cerevisiae* strain W303α using primers TP907/TP1497 with 5'*BamH*I and 3' *Spe*I, respectively. The intron of Mei4 was got rid of using overlapping PCR with primers TP1936 and TP1937. The Mei4 PCR product was introduced into the pFastbac1 vector with an N-terminal Flag-tag at the *BamH*I and *Spe*I sites to yield pTP1639. pTP1639 was made into bacmid pTP1640.

REC114 was PCR amplified from wild-type *S. cerevisiae* strain W303α using primers TP909/TP1526 with 5' *BamH*I and 3' *Spe*I, respectively. The intron of *REC114* was removed using overlapping PCR with primers TP1934 and TP1935. Mei4 coding sequence PCR product was introduced into the pFastbac1 vector with *BamH*I and *Spe*I sites to yield pTP1334. pTP1334 was made into b3acmid pTP1345. All bacmids were used to make baculvirus according to the Bac-to-Bac instructions from manufacturers (Invitrogen).

Constructs to express the yeast MRX complex and Exo1 in insect cells were described previously. (Bhaskara, Dupre et al. 2007; Nicolette, Lee et al. 2010)

PROTEIN EXPRESSION AND PURIFICATION

Protein Expression and Purification of the Spo11 complex

The wild-type Spo11, Ski8, Rec102, Rec104 complex was expressed from baculovirus made from pTP941, pTP921, pTP988, and pTP1344 in Sf21 insect cells. All purification steps were performed at 4℃. Cells were thawed on ice, lysed by homogenization and sonicated three times for 20 seconds in Ni A buffer (500 mM KCl, 50 mM KH_2PO_4 pH, 10% glycerol, 10 mM imidazole, 20 mM 2-mercaptoethanol) containing 0.5% tween-20 and 1 mM PMSF. The lysate was centrifuged at 35,000 RPM for one hour. The supernatant was removed and loaded onto a Ni-NTA (Qiagen) column. The resin was washed with 10 ml Ni A buffer, 10 ml low salt Ni A buffer (100 mM KCl, 50 mM KH_2PO_4 pH, 10% glycerol, 10 mM imidazole, 20 mM 2-mercaptoethanol), 10 ml 10% Ni B buffer (500 mM KCl, 50 mM KH_2PO_4 pH, 10% glycerol, 250 mM imidazole, 20 mM 2-mercaptoethanol), and the protein complex was eluted with a 12 ml gradient of Ni B buffer from 10% to 100%. Peak fractions from Ni-NTA column were applied to an amylose column (New England Biolabs, NEB). The resin was washed with 10 ml A buffer (100 mM NaCl, 25 mM Tris pH8.0, 10% glycerol, 1 mM DTT), and eluted with 10 ml of A buffer containing 10 mM maltose. The peak fractions from amylose column were loaded onto Anti-Flag M2 agarose resin (Sigma). The resin was washed with 10 ml of 500 mM LiCl, followed by a wash with 10 ml A buffer, and the protein complex was eluted with 5 ml of A buffer containing 0.1 mg/ml Flag peptide (Sigma). The peptide was allowed to flow to the column bed and paused for 20 min, and then the elution was continued. The protein complex was collected in 0.5 ml fractions, aliquoted, and fast frozen in liquid nitrogen, and stored in -80℃. The protein complex containing the

mutant Spo11 (Y135F) was expressed from baculovirus made from pTP967, pTP921, pTP988, and pTP1344 in Sf21 insect cells, and purified in the same way.

Protein Expression and Purification of HisMBP-Sae2 Protein, MRX complex and Exo1

His-MBP-Sae2 was expressed in the ArcticExpress strain (Invitrogen) of *E. coli* cells. The expression and purification of recombinant Sae2 protein was described previously (Lengsfeld, Rattray et al. 2007). Briefly, the cell lysate was applied onto Amylose agarose resin (NEB). The maltose elution from the Amylose column was loaded onto SP-Sepharose resin (G.E.). The high salt elution from the SP-Sepharose column was loaded onto Ni-NTA (Qiagen) resin. The elution from Ni-NTA resin was then applied to two tandem SP-Sepharose HiTrap columns (G.E.). The peak fraction was then loaded onto a Superdex-200 gel filtration column (G.E.). Monomeric Sae2 was separated from the dimeric and multimeric peaks and eluted in fraction #27~#29. Fraction #28 was used for all experiments described below.

The Mre11/Rad50/Xrs2 complex was expressed from baculovirus made from bacmids pTP404, pTP684 and pTP701 in Sf21 insect cells, as was previously described. (Bhaskara, Dupre et al. 2007). For purification, cell lysate was precipitated with ammonium sulfate, and then the protein complex was purified after going through Ni-NTA (Qiagen) resin, 1ml HiTrap Heparin column (G.E.), and Anti-Flag M2 agarose resin (Sigma) as described previously (Bhaskara, Dupre et al. 2007).

Exo1 was expressed and purified as described (Nicolette, Lee et al. 2010). It was expressed from baculovirus in Sf21 insect cells, and purified after the cell lysate was applied to SP-Sepharose resin (G.E.). The high salt elution from the SP-Sephasrose

Table 2.2 Yeast strains used in this study.

Strain	Geneotype	Strain construction
Strains	for recombination assay[a]	
TP3226	*sae2::kanMX*	
TP3330	*sae2::kanMX* (pRS313)	TP3226+pRS313
TP3231	*sae2::kanMX* (pTP1350, wild-type *His-MBP-Sae2*)	TP3226+pTP1350
TP4674	*sae2::kanMX* (pTP2110, His-MBP-*sae2 (S84A/E85G/D86G/F87L)*)	TP3226+pTP2110
TP3387	*sae2::kanMX*(pTP1477,His-MBP-*sae2 (L25P)*)	TP3226+pTP1477
TP3384	*sae2::kanMX* (pTP1382,His-MBP-*sae2 (E226A)*)	TP3226+pTP1382
TP3385	*sae2::kanMX* (pTP1474, His-MBP-*sae2 (N212G)*)	TP3226+pTP1474
TP3604	*sae2::kanMX* (pTP1518, His-MBP-*sae2 (I205G)*)	TP3226+pTP1518
Strains	for TCA extraction[b]	
TP2012	*sae2::kanMX*	
TP3540	*sae2::kanMX* (pRS425)	TP2012+pRS425
TP3392	*sae2::kanMX* (pLEU2promFlag-SAE2, wild-type Flag-*Sae2*)	TP2012+ pLEU2promFlag-SAE2[d]
TP4672	*sae2::kanMX* (pTP2109, Flag-*sae2 (S84A/E85G/D86G/F87L)*)	TP2012+pTP2109
TP3932	*sae2::kanMX* (pTP1802, Flag-*sae2 (E207A/D208Q)*)	TP2012+pTP1802
TP3924	*sae2::kanMX* (pTP1517, Flag-*sae2 (L25P)*)	TP2012+pTP1517
TP4420	*sae2::kanMX* (pTP2009, Flag-*sae2 (D193A)*)	TP2012+pTP2009
TP4444	*sae2::kanMX* (pTP2011, Flag-*sae2 (W204L)*)	TP2012+pTP2011
TP4419	*sae2::kanMX* (pTP2008, Flag-*sae2 (I205G)*)	TP2012+pTP2008
Strains	for sporulation[c]	
TP2780	TP1158 *spo11:: MBP-Spo11*	
TP2939	TP1158 *spo11::Gal4BD-MBP-Spo11*	
TP2134	TP1158 *spo11::kanMX*	
TP4310	TP1745 *spo11::kanMX*	
TP4404	TP1745 *spo11::kanMX*	pRS316
TP4514	Diploid of TP2780 and TP4404	
TP4515	Diploid of TP2939 and TP4404	
TP1860	TP1745	pRS316
TP2564	Diploid of TP1158 and TP1860	
TP4432	Diploid of TP2134 and TP4404	
Strains	for MMS sensitivity assay[a]	
TP3367	*yKu:: kanMX*	
TP5007	*sae2::URA3*	
TP5008	*yKu:: kanMX sae2::URA3*	

TP3308	*SAE2::sae2 (S267E)*
TP3327	*SAE2::sae2 (S267A)*
TP3368	*SAE2::sae2 (S267E) yKu:: kanMX*
TP3369	*SAE2::sae2 (S267A) yKu:: kanMX*

[a] Strains isogenic to ALE94 (*MATa ade5-1 his7-2 leu2-3 112:p305L3(LEU2) trp1-289 ura3-D lys2:AluIR*) (Gift from K. Lobachev)

[b] Strains isogenic to BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*)

[c] TP1158 are isogenic to BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*). TP1745 is isogenic to W1588-4A (W303β with corrected *RAD5*, aka LSY0678), that is *MATα RAD5 leu2-3, 113 trp1-1, ura3, can1, his3-11, 15 ade2-1*.

[d] Gift from A. Rattray

column was loaded onto Anti-Flag M2 agarose resin (Sigma), washed with 10 ml 500 mM LiCl, and eluted with 5 ml of A buffer containing 0.1 mg/ml Flag peptide (Sigma).

YEAST STRAINS

All strains used in this study are listed in Table 2.2. All strains for the recombination rate between inverted repeats assay (Lys2 conversion assay) are isogenic to ALE94 (*MATa ade5-1 his7-2 leu2-3 112:p305L3(LEU2) trp1-289 ura3-D lys2:AluIR*) (Gift from K. Lobachev). All strains for the TCA extraction are isogenic to BY4741 (*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*). The *SAE2* gene and was deleted using the *sae2::kanMX* cassette made in the Yeast Deletion Collection (http://www-sequence.stanford.edu:16080/group/yeast_deletion_project), or *using sae2::URA3* cassette made by T. Paull in pTP1653. The *yKu* gene was deleted using the *yku::kanMX* cassette from ATCC. The site-directed mutagenesis of the *SAE2* gene on the chromosome, and the *Gal4BD-MBP-SPO11* and *MBP-SPO11* gene replacement on the chromosome was performed using the PCR-based gene disruption method developed by R. Reid (Reid, Sunjevaric et al. 2002).

SAE2 GEL MOBILITY SHIFT ASSAY

The DNA substrate was prepared as described previously, by PCR amplification of a 249bp fragment in the presence of [α-^{32}P] dATP (Lengsfeld, Rattray et al. 2007). The PCR product was separated on a 1% agarose gel and extracted with UltraFree-DA columns (Millipore). Gel mobility shift assays were performed in a volume of 10μL for Sae2. The final reaction concentrations were 25mM 3-(N-morpholino) propanesulfonic acid, pH 7.0 (MOPS), 2mM DTT, 10mM ethylenediaminetetraacetic acid (EDTA), 100mM NaCl with 1 ng DNA substrate. The reactions were incubated on ice for 20

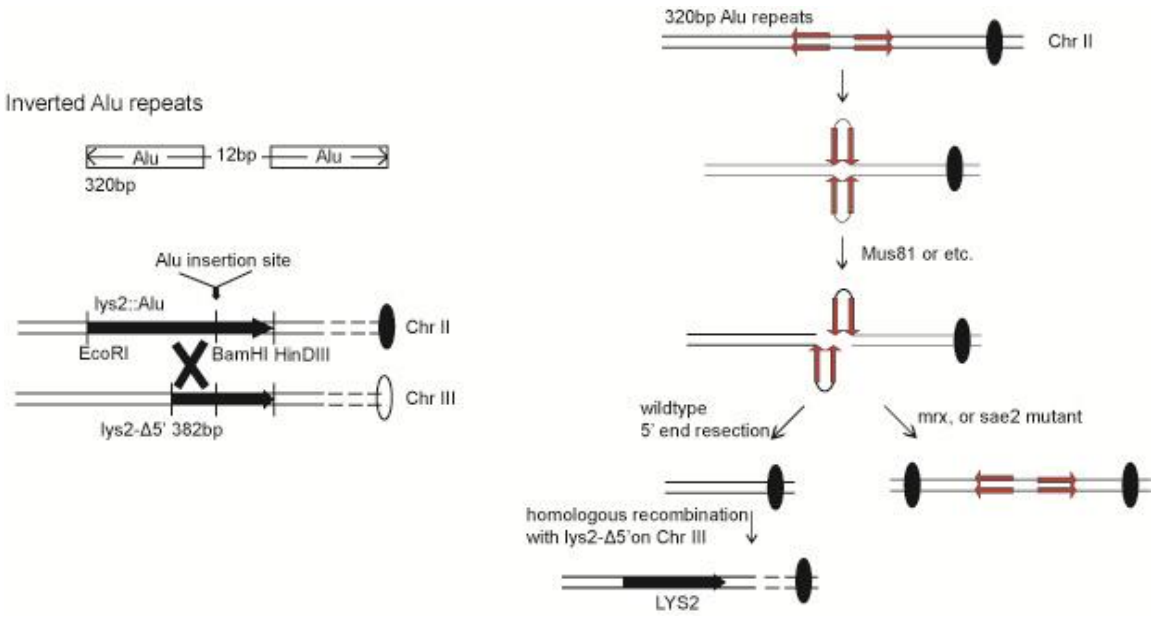


Figure 2.1 Schematic representation of the Lys2 conversion assay.

In ALE94 (Lobachev, Gordenin et al. 2002), 320bp Alu inverted repeats are present in the *Lys2* gene on the Chromosome II in an inverted orientation. Inverted repeats can form a cruciform structure *in vivo*, which is a vulnerable structure. A cruciform can be cleaved by Mus81 or other enzymes, in an MRX and Sae2-independent manner (Cote and Lewis 2008). In wild-type cells, MRX and Sae2 remove the hairpin structure and prepare DNA ends for long range resection and homologous recombination. In *mrx* or *sae2* mutant cells, stable hairpin structures lead to DNA replication of the hairpin and formation of large palindromes in the chromosome. (Figure adapted from (Lobachev, Gordenin et al. 2002; Cote and Lewis 2008))

minutes with Sae2 protein concentrations as indicated. The gel mobility shift assays with the Sae2 proteins were resolved in an 8% 37.5:1 native PAGE bis-tris gel. All gel mobility shift assays were analyzed by phosphorimager (Typhoon).

OLIGONUCLEOTIDE SUBSTRATE PREPARATION

All 5' [^{32}P] labeled nuclease substrates(TP74/TP1152, or TP2553) were labeled with T4 polynucleotide kinase (PNK) (NEB). To anneal duplex substrates, the labeled strands were boiled and slowly cooled to RT in 100 mM NaCl and 10 mM Tris, pH 8.0 and 1 mM EDTA.

NUCLEASE ASSAY

Endonuclease Hairpin Assay: Reactions with either TP74/TP1152 (flap DNA) or TP2553 (hairpin DNA) were performed in 10μL total volumne with 25 mM MOPS, 2 mM *DTT, 50* mM NaCl, 5 mM $MgCl_2$ and 1 mM ATP for 30 minutes at 30° C.
TP74/TP1152 can form a flap with a 4 bp 5' overhang, a 31 bp stem, and a 15 bp flap. TP2553 can form a self-annealing hairpin with a 16 bp stem, an 8 nt self-annealing homology and a 18 nt gap.

All nuclease reactions were resolved in 20% denaturing polyacyralmide gels (20% acrylamide, 7.5 urea, 1X TBE), which were analyzed by phosphorimager (Typhoon).

RECOMBINATION RATE BETWEEN INVERTED REPEATS

Rates of recombination induced by inverted Alu repeats were determined as previously described (Lobachev, Gordenin et al. 2002). Briefly, the SAE2 gene was deleted using the *sae2::kanMX* cassette from Δ*sae2* in BY4741 (MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0, Yeast Deletion set from Invitrogen) in the ALE94 strain (gift from K.

Lobachev). SAE2 wild-type and mutant genes were transformed into this strain on CEN ARS HIS-containing plasmids (derivatives of pRS313). 12 colonies of each subsequent strain were grown in synthetic medium lacking histidine, grown to log-phase, and each culture was washed three times before plating of 0.1 to 2 OD A600 on synthetic medium lacking lysine. Control plates of systhetic medium lacking histidine were done in parallel to count viable cells. The rate of recombination was calculated for each strain by fluctuation analysis (Spell and Jinks-Robertson 2004) and the standard deviation is as shown.

RESECTION ASSAY

All resection assays were carried out with the DNA plasmid substrate pNO1 (a 4.4-kb derivative of pBR322) linearized with *Sph*I-HF. Resection reactions contained 5 ng (0.17 nM pNO1) plasmid DNA, 25 mM MOPS, pH 7.0, 60 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, and 0.5 mM ATP, and were performed at 30°C for one hour.

Reactions were stopped by the addition of 0.2% (w/v) SDS and 10mM EDTA, incubated with 1 μg proteinase K at 37 °C for 20 min, analyzed on 1% agarose gels run in 1× TAE (Tris-acetate-EDTA, containing 40 mM Tris-acetate, and 1 mM EDTA) buffer, and visualized with SYBR green (Invitrogen). Resection assays were further analyzed by nondenaturing Southern hybridization, in which agarose gels were first washed into 20× SSC (3 M NaCl, and 0.3 M sodium citrate), and then DNA was transferred by capillary action onto nylon membranes (NEN) overnight in 20× SSC. Membranes were fixed by UV, and probed with RNA complementary to 3′ strand of the linearized pNO1 substrate in a 1 kb region adjacent to the *Sph*I site. The probes were internally labeled with [α-^{32}P] CTP (NEN) using Riboprobe System T7 (Promega) and purified with RNeasy extraction (Qiagen) according to the manufacturer instructions.

QUANTITATIVE PCR

Resection assays were carried out as described above, but stopped with a final concentration of 0.01% SDS. Quantitative PCR was performed as described previously (Nicolette, Lee et al. 2010). The reactions were diluted 20-fold and half of the mixture was digested overnight at 37°C with 4 units of *Nci*I (NEB) and the other half was incubated in the same buffer without the enzyme at 37°C overnight. 1 µl of digested or undigested DNA sample was used as a template in a 25 µl reaction with 0.5 µM of each primer, 0.2 µM probe, and 1X Taqman universal master mix (ABI). Q-PCR reactions were performed on 7900HT Fast Real-Time PCR System (ABI) under standard thermal cycling conditions for 30 cycles. Results were analyzed with SDS2.3 (ABI). For each sample, a ΔCT was calculated by subtracting the CT value of the undigested sample from the CT value of the *Nci*I-digested sample. The percentage of ssDNA was determined using this equation: ssDNA%=1/(2^(ΔCT-1)+0.5)*100. Primers and probes used for the analysis of the 29 nt site were: TP2493, TP2494, and TP2495. Primers and probes used for the analysis of the 1025 nt site were: TP2516, TP2517, and TP2518.

PROTEIN EXTRACT ANALYSIS

To analyze the expression level of Sae2 mutants in yeast, trichloroacetic acid (TCA)-extracted whole cell lysates were prepared (Kim, Vijayakumar et al. 2008). and expression of different sae2 mutants was analyzed by Western blot using a monoclonal anti-Flag (M2) antibody (Sigma). To investigate the phosphorylation level of Sae2 protein after DNA damage, whole cells lysates were prepared in NP-40 buffer (25 mM Tris pH7.4, 1mM EDTA, 1 mM DTT, 500 mM NaCl, 0.5% NP-40, 10% glycerol) and immunoprecipitated with anti-Flag M2 agarose beads (Sigma) after MMS treatment of the yeast culture for 4 hours at the concentration of 0.03%. Protein isolated from the

lysate was further analyzed by western blot using anti-Sae2 (custom-made antibody from Precision) or anti-phospho-SQ/TQ antibodies (Cell Signaling).

RANDOM SPORE ANALYSIS

Random spore analysis for wild-type, MBP-Spo11 and, Gal4BD-MBP-Spo11 diploid strains were performed as described previously (Rockmill, Lambie et al. 1991). 3 single colonies are patched onto YPD plate and grow at 30°C for 12 to 16 hours. A matchhead equivalent of yeast cells were inoculated into Sporulation Medium (1% KOAc, 0.0225% glulcose). The cultures stayed at RT (25°C) for ten days. Cells were counted under microscope using a hemocytometer. 1 O.D. A600 cells were collected and treated with β-glucurodinase (500 units) for 1 hour and were incubated with glass beads on a rotator for 1 hour to disrupt the cell wall. Serial dilution was performed and cells are plated on synthetic medium plates without arginine (with 60 μg/ml canavanine) to select for *can1* haploid cells a countable colony number. Sporulation efficiency was calculated for all strains and standard deviation is as shown.

SPO11 CLEAVAGE AND BINDING ASSAY

Gel Mobility Shift Assay

The oligonucleotide DNA substrate was prepared by labeling TP1524 or TP74 with 5' [^{32}P] with T4 polynucleotide kinase (PNK) (NEB), and unincorporated nucleotide was removed with the Nucleotide Removal Kit (Qiagen). To anneal TP1524 with TP1525, or TP74 with TP124, the labeled strands were boiled, incubated at 55° C in 100 mM NaCl and 10 mM Tris, pH 8.0 and 1 mM EDTA for 30 min, and cooled down to RT (25° C) slowly. TP1524/TP1525 contains one *GAL4* consensus site. TP74/TP124 does not contain a *GAL4* consensus site. Gel mobility shift assays were performed in a volume

of 10μL for Spo11. The final reaction concentrations were 25mM 3 - (N-morpholino) propanesulfonic acid, pH 7.0 (MOPS), 2 mM DTT, 10 mM ethylenediaminetetraacetic acid (EDTA), 80mM NaCl. The reactions were incubated on ice for 20 minutes with recombinant Gal4BD-MBP-Spo11 complex, or MBP-Spo11 complex, and MRX complex, Sae2 protein amount as indicated. The gel mobility shift assays were resolved in 0.7% agarose gel in 0.5X TBE (Tris/Borate/EDTA) buffer. All gel mobility shift assays were analyzed by phosphorimager (Typhoon).

Plasmid or Oligonucleotide Cleavage Assay

Cleavage assays of Spo11 were performed either with oligonucleotide substrates (TP1524/TP1525, with one *GAL4* consensus site) as described in Gel Shift Assay above, or with a supercoiled plasmid pTP407 containing a *GAL1/10* region in 10μL total with 25 mM MOPS, 1 mM DTT, 100 mM NaCl, 5 mM $MgCl_2$ and 1 mM ATP for one hour at 30 ° C.

Cleavage assays with oligonucleotide substrates were resolved in 20% denaturing polyacyralmide gels (20% acrylamide, 7.5 urea, 1X TBE), which were analyzed by phosphorimager (Typhoon). Cleavage assays with plasmid substrates were resolved in 0.8% agarose gels (1X TAE), which were stained with SYBR green and analyzed by phosphorimager (Typhoon).

CHAPTER 3 SPO11 COMPLEX EXPRESSION AND PURIFICATION

INTRODUCTION

Homologous recombination occurs during prophase I of meiosis. It plays a dual role by shuffling information between homologous chromosomes, so as to create a more diversified genetic background in the progeny, and also to ensure the proper segregation of homologs during the first meiotic division. Natural meiotic DSB sites do not distribute evenly along the chromosomes. Numerous factors, both local ones and global ones, contribute to this non-random distribution of DSBs into "hot spots". Locally, an open chromatin structure is necessary for meiotic DSB formation. Almost all known natural DSB sites are also susceptible to DNaseI and micrococcal nuclease (MNase) (Ohta, Shibata et al. 1994; Wu and Lichten 1994; Fan and Petes 1996; Keeney and Kleckner 1996). However, chromatin structure is not the only contributor to DSB formation, since not all nuclease-sensitive regions are DSB sites. Natural DSB sites are also more likely to occur in promoter regions (Wu and Lichten 1994; Baudat and Nicolas 1997). Globally, genome-wide mapping of meiotic DSBs reveal that there are "cold" and "hot" domains on chromosomes for DSBs. So far, very little is known for what makes a domain "cold" or "hot".

In budding yeast, Spo11 is the catalyst of meiotic DSB formation. *SPO11* was first identified in a screen for temperature-sensitive mutants that failed in ascospore formation (Esposito, Frink et al. 1972; Esposito and Esposito 1974). The eukaryotic members of Spo11 all share sequence similarity with the smaller subunit of TopoVI (Top6A), a type II topoisomerase from archaea (Ajimura, Leem et al. 1993; Bergerat, de Massy et al. 1997; Keeney, Giroux et al. 1997). Although eukaryotic Spo11 has never

been shown to cleave DNA directly, Spo11-DNA adducts were observed in *rad50S*, *mre11S*, and Δ*sae2* yeast strains, while no such adducts have ever been observed in wild-type strains. In budding yeast, the profile of identified components required for meiotic DSB formation is most complete. Spo11 needs Ski8, Rec102, Rec104, Mer2, Mei4, Rec114 and MRX to perform the cleavage. Spo11-DNA adducts are further processed by single-stranded endonucleolytic cleavage, for which MRX and Sae2 are essential. The length of oligonucleotides attached to Spo11 in the cleavage products varies from species to species. In budding yeast or mice, an asymmetric cleavage results in two kinds of oligonucleotides (~21-37nt or ≤12nt in yeast, and ~12-26nt or ~28-34nt in mice) covalently attached to Spo11. In fission yeast, Spo11-DNA adducts are processed by a symmetric cleavage which produces a single size class of Rec12 (Spo11)-oligonucleotides (~13-29nt) complexes (Rothenberg, Kohli et al. 2009).

Peciña *et al.* fused the DNA binding domain of Gal4 protein (Gal4BD) to the N-terminus of Spo11, and demonstrated that this fusion protein could generate targeted DSBs (Pecina, Smith et al. 2002). The Gal4 transcription factor can bind to a consensus 17 base pair ($CGGN_{11}CCG$) recognition site (Johnston 1987). Expression of this Gal4BD-Spo11 protein not only complemented the sporulation defect of Δ*spo11* strains, but also stimulated meiotic DSB formation and recombination close to UAS_{GAL} sites. Fukuda et al. further characterized this targeted DSB formation, and showed that such DSBs are still dependent on all the known DSB accessory factors, and that known hot sites without GAL4 binding consensus motifs are also still used in this strain (Fukuda, Kugou et al. 2008).

Deletion of any component among Spo11, Ski8, Rec102, Rec104, Mer2, Mei4, Rec114, and Mre11/Rad50/Xrs2 results in a sporulation defect. However, the specific

role of any of them, other than Spo11, remains obscure. Spo11 has long been suggested to be the catalyst of meiotic DSB events, however, it has never been demonstrated to cleave DNA directly. Spo11 is difficult to express in recombinant form and it precipitates easily in cell lysates (Wu, Gao et al. 2004). I fused a Maltose-Binding-Protein to the N-terminus of Spo11 to overcome the expression difficulty by significantly increasing the solubility of Spo11. I also attached a Gal4BD tag to the N-terminus of MBP-Spo11 as well, so as to reconstitute targeted DSBs formation *in vitro*.

RESULTS

To examine whether Gal4BD-MBP-Spo11 can recapitulate wild-type Spo11 activity in yeast, I made a strain with a *GAL4BD-MBP-SPO11* replacing the wild-type *SPO11* gene in the chromosome, and another strain with an *MBP-SPO11* replacing the wild-type *SPO11* gene in the chromosome. The *Gal4BD-MBP-SPO11* and *MBP-SPO11* gene replacement was verified by southern blot. As shown in Figure 3.1, I analyzed the sporulation efficiency of wt $SPO11^{+/-}$, $spo11^{-/-}$, $MBP\text{-}SPO11^{+/-}$, $GAL4BD\text{-}MBP\text{-}SPO11^{+/-}$ strains using random spore analysis. Diploid strains used in this analysis are *can1/CAN1* heterozygotes. After a serial dilution, cells were plated on synthetic medium plates without arginine (with 60 μg/ml canavanine) to select for *can1* haploids. The ratio of sporulation of the $GAL4BD\text{-}MBP\text{-}SPO11^{+/-}$ strain and $MBP\text{-}SPO11^{+/-}$ strain is similar to that of the wild-type strain.

According to previous genetic and physical evidence, I made the hypothesis that Spo11, Ski8, Rec102 and Rec104 forms a complex, and it is very likely the core component of DSB formation during meiosis. I made the constructs and bacmids to express recombinant Gal4BD-MBP-Spo11, Ski8-HIS, Rec102-HIS, and Rec104-FLAG (Gal4BD-Spo11 complex), and MBP-Spo11, Ski8-HIS, Rec102-HIS, and Rec104-FLAG

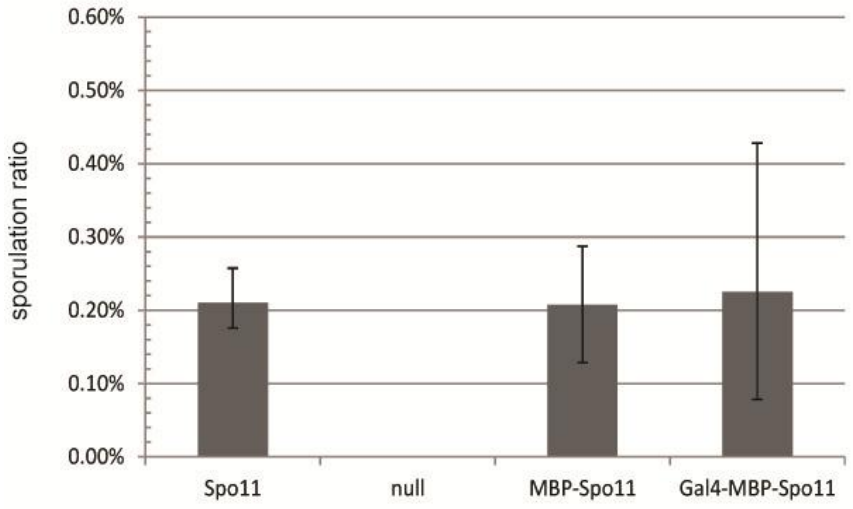


Figure 3.1 Random spore analysis of recombinant Spo11.

The *MBP-SPO11*$^{+/-}$ strain, *GAL4BD-MBP-SPO11*$^{+/-}$ strain and wild-type *SPO11*$^{+/-}$ strain have similar ratio of sporulation. Diploid strains in this analysis are *can1/CAN1* heterozygotes. Cells were treated with β-glucurodinase (500 units) for 1 hour and were incubated with glass beads on a rotator for 1 hour to disrupt the cell wall. Cells were plated on synthetic medium plates without arginine (with 60 μg/ml canavanine) to select for *can1* haploids after a serial dilution to get a countable number. The average was calculated for three independent analyses, and the standard deviation is as shown. The lower limit for random spore analysis performed here was 0.01%.

(MBP-Spo11 complex) in insect cells. All four proteins were expressed simultaneously in Sf21 insect cells. The protein complex was purified by sequential Ni-NTA, amylose and anti-Flag affinity M2 columns. The eluted proteins from these columns at each step of the purification were analyzed by western blot with anti-Flag, anti-HIS, anti-MBP antibodies (Figure 3.2). Spo11, Ski8, Rec102 and Rec104 did form a complex as we expected. The protein complex containing Spo11 (Y135F) was expressed and purified in the same way. This putative catalytic-deficient Spo11 mutant did not affect the protein interaction between Spo11, Ski8, Rec102, and Rec104.

To test whether the Spo11/Ski8/Rec102/Rec104 complex I purified is functional or not, I first studied the DNA binding ability of this complex (Figure 3.3 A). I first used an oligonucleotide containing one *GAL4* consensus sequence (TP1524/1525) as a binding substrate. I found that Gal4BD-MBP-Spo11 complex forms a larger complex with DNA, as compared to DNA substrate alone. The Spo11 (Y135F) mutant is almost equally capable in DNA binding. I also used an oligonucleotide of similar length but without the *GAL4* consensus sequence (TP74/124) as a substrate for the gel shift assay. Surprisingly, the Gal4BD-MBP-Spo11 complex also bound well with this substrate. However, MBP-Spo11 complex did not bind as efficiently as Gal4BD-MBP-Spo11 did with either substrate. This result indicated that the binding between the Gal4BD-MBP-Spo11 complex and DNA does not depend on the *GAL4* consensus site in the oligonucleotide substrate, but rather on the presence of GAL4BD at the N-terminus of Spo11.

I further tested the activity of Spo11 complex to generate DSBs. I used two kinds of substrates. The first one is the same oligonucleotide I used in the gel mobility shift assay. The Spo11 complex did not cleave the oligonucleotide substrate containing one *GAL4* site, even in the presence of MRX and Sae2 (data not shown). The other substrate I

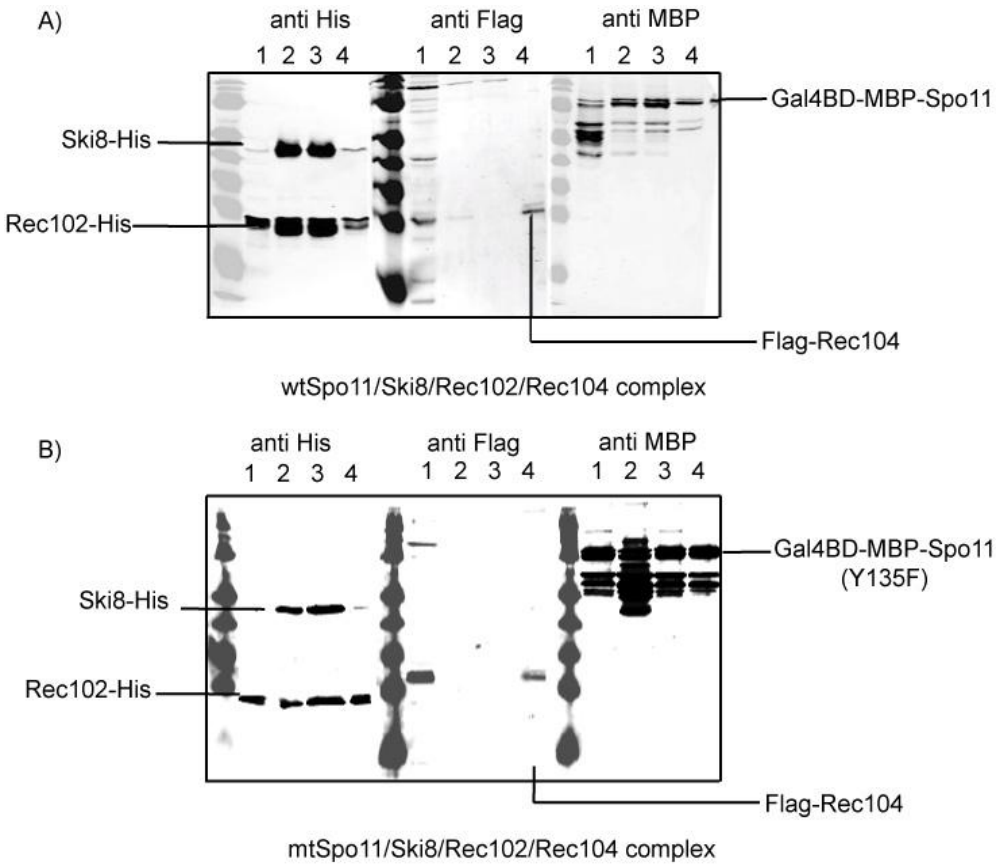


Figure 3.2 Western blot of recombinant Spo11 wt complex (A) and Spo11(Y135F) complex (B).

A) Western blot of recombinant Spo11 complex (1. Ni-NTA flow through; 2. Ni-NTA elution peak fraction; 3. Anti-Flag beads flow through; 4. Anti-flag beads elution peak fraction). B) Western blot of recombinant Spo11 (Y135F) complex (1. Ni-NTA flow through; 2. Ni-NTA elution peak fraction; 3. Anti-Flag beads flow through; 4. Anti-flag beads elution peak fraction). The Spo11 complex was purified after the insect cell lysates were applied sequentially onto Ni-NTA column, Amylose column and Anti-Flag agarose resin as described in Methods. The Spo11/Ski8/Rec102/Rec04 complexes were analyzed in 12% SDS-PAGE gels, followed by western blot with the antibodies as indicated.

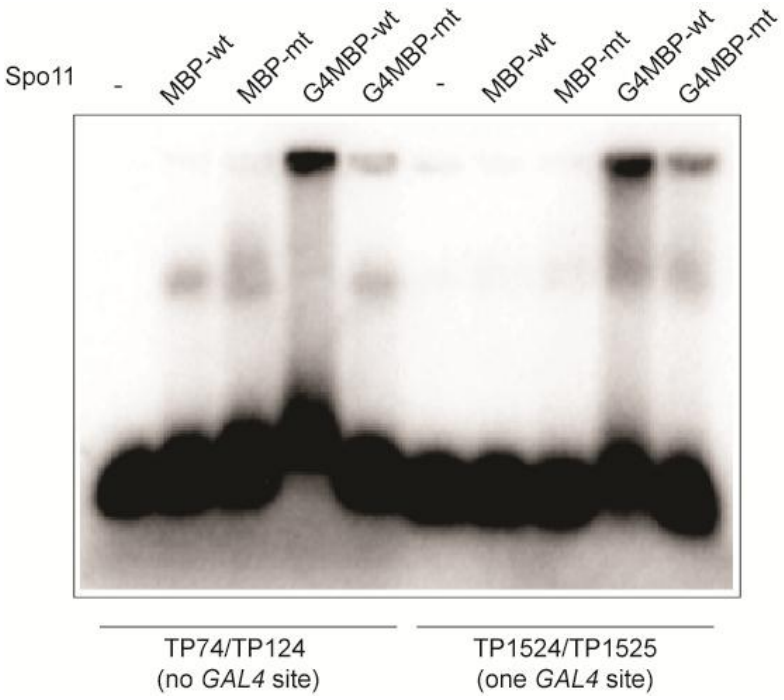


Figure 3.3 Gel shift assay of recombinant Spo11 complex.

The Gal4BD-MBP-Spo11 complex binds oligonucleotide substrates with or without the *GAL4* consensus site. MBP-Spo11 complexes (wild-type (MBP-wt) and Y135F (MBP-mt)), and Gal4BD-MBP-Spo11 complexes (wild-type (G4MBP-wt) and Y135F (G4MBP-mt)) were tested in a gel mobility shift assay with [^{32}P]-labeled 50 bp oligonucleotide substrates containing a *GAL4* consensus sequence (TP1524/TP1525) or no *GAL4* site (TP74/TP124). Protein-DNA complexes were resolved in a 0.7% agarose gel run in 0.5X TBE (Tris/Borate/EDTA) buffer.

used is a supercoiled plasmid (pTP407) containing the GAL1/10 upstream activator sequence (UAS) region, which has several Gal4 consensus sites. The supercoiled plasmid DNA is either negatively supercoiled or positively supercoiled after treatment with the Hmf B protein. In this analysis, which was performed with the assistance from J. Cuenca, we obtained a preliminary result that the Gal4BD-MBP-Spo11 complex created a break in this plasmid DNA, while the catalytic-deficient mutant Spo11 (Y135F) complex did not. Such catalytic activity was further stimulated by MRX and Sae2 (Figure 3.4).

CONCLUSIONS

I have successfully expressed and purified recombinant Gal4BD-MBP-Spo11 or MBP-Spo11, together with its close partner Ski8, Rec102, and Rec104. Our result indicated that Gal4BD-MBP Spo11 complex bound strongly to the DNA oligonucleotide substrate with or without the *GAL4* consensus site. The binding between Spo11 and DNA partially depends on the Gal4BD domain, because MBP-Spo11 complex does not bind as well with same substrates.

However, the binding between the Spo11 complex and DNA is not enough to generate DSB in the oligonucleotide. One possibility is that the oligonucleotide DNA substrate I used is not long enough. A previous study illustrated that targeted meiotic DSBs form near Gal4 sites, instead directly at the sites (Johnston 1987). I may need a much longer substrate to observe the activity of Spo11. The other possibility is that, instead of linear DNA, some secondary structure of DNA is essential feature of Spo11 substrates. I tested this hypothesis by using a supercoiled DNA with a GAL1/10 region. Within that region, there are several Gal4 sites. I observed DSBs occurrence in the presence of the wild-type Gal4BD-MBP-Spo11 complex and not with the mutant Spo11 (Y135F) complex. Addition of proteinase K had no effect on Spo11 activity. Our

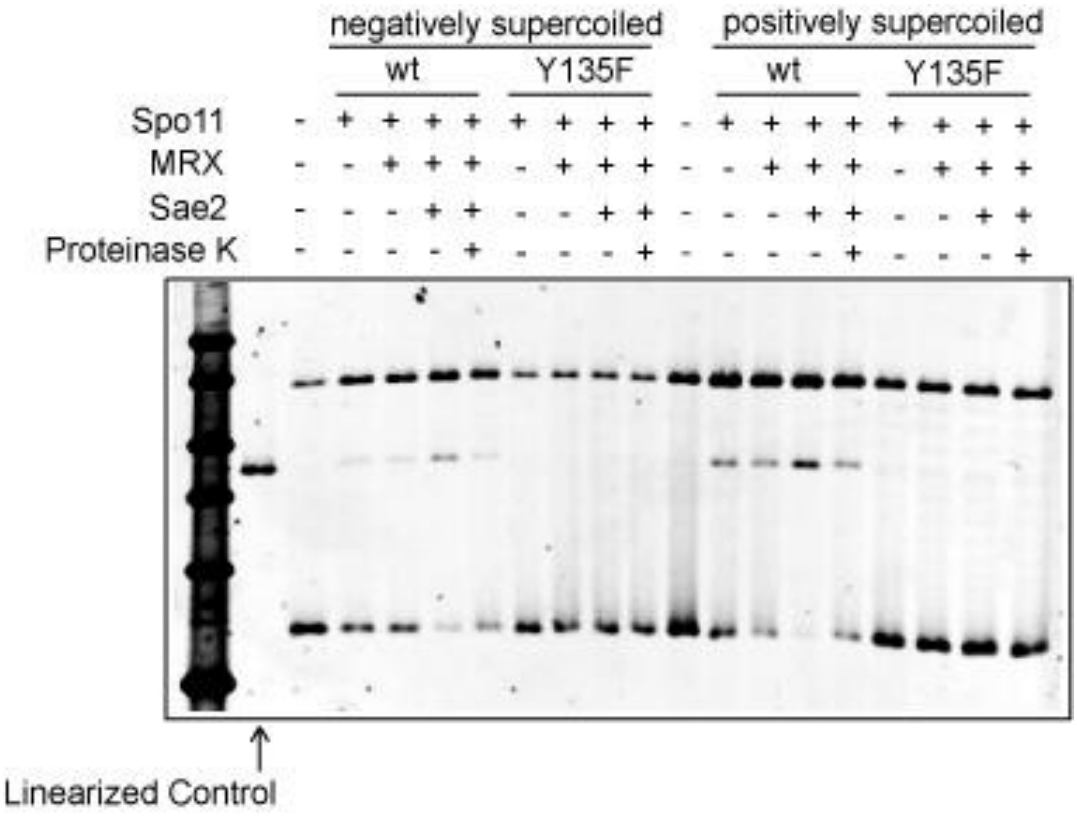


Figure 3.4 Cleavage assay of recombinant Spo11 complex.

Purified recombinant wild-type Gal4BD-MBP-Spo11 complex cleaves supercoiled plasmid DNA containing a GAL1/10 region. The complex containing catalytic deficient mutant Spo11 (Y135F) does not show such activity. Addition of MRX and Sae2 creates a small stimulation on the cleavage activity of wild-type Spo11 complex, while addition of proteinase K does not. Products were resolved in a 0.8% agarose gel, and stained with SYBR green.

preliminary result showed that the addition of MRX and Sae2 created a small stimulation on the cleavage activity of wild-type Spo11 complex.

Presently, the Spo11 complex I purified is clearly catalytically active, but it has not fully recapitulated its activity as previously observed *in vivo* (Fukuda, Kugou et al. 2008). With the assistance of J. Cuenca, we found that the GAL4 consensus sites on the supercoiled plasmid substrates we used in the cleavage assay (Figure 3.4) are not required, and MBP-Spo11 complex failed to cleave the same supercoiled plasmid substrate as the Gal4BD-MBP-Spo11 did (data not shown). This suggests that the binding between the Spo11 complex and DNA substrates through the Gal4 domain may have overcome the requirement for other components essential *in vivo* (MRX, Sae2, Mer2/Mei4/Rec114), even though this apparently did not occur with expression of GAL4BD-Spo11 *in vivo*. One possibility is that the MBP domain facilitates DNA binding that is independent of the other accessory factors. Alternatively the supercoiled plasmid substrate I used does not accurately mimic the natural substrate. *In vivo*, it is possible that the chromosomal DNA has unusual topology that it has a specialized chromatin structure, or it is bound by polyamines (Pingoud, Urbanke et al. 1984). It is also possible that other components like Mer2, Mei4, Rec114, which were found to be essential for meiotic DSB formation *in vivo*, are also required to prepare the DNA substrate to be a suitable one, or to prepare the Spo11 complex to be in active conformation. I have made all constructs and bacmids to express these three proteins and the effect of these complexes on Spo11 activity are currently being investigated.

CHAPTER 4 CHARACTERIZATION OF THE ENDONUCLEASE ACTIVITY OF SAE2

INTRODUCTION

In *S. cerevisiae*, MRX and Sae2 play an important role in DSB repair during meiosis and during vegetative growth. Sae2 has long been regarded as the fourth component of the MRX complex because *rad50S*, *mre11S*, and *Δsae2* strains share similar phenotypes upon meiotic DSBs. Spo11-DNA adducts accumulate in *rad50S* and *Δsae2* stains during meiotic prophase I, which block DSB processing and sporulation. In vegetatively growing cells, in the presence of a DSB within an inverted repeat on the chromosome, aberrant chromosomes with a hairpin structure on the end or large palindrome duplication of chromosomes accumulate in *rad50S*, *mre11S* and *Δsae2* strains. MRX and Sae2 were suggested to remove such hairpin structures, as well as protein-DNA adducts formed during radiation or chemical exposure, and to prepare the DNA ends for further resection by Exo1, or Sgs1/Dna2 (Lobachev, Gordenin et al. 2002).

Lengsfeld *et al.* showed that Sae2 exhibits endonuclease activity *in vitro* (Lengsfeld, Rattray et al. 2007). Sae2 prefers single-stranded DNA and single-stranded/double-stranded DNA junctions as its substrate *in vitro*. Sae2 also has a preference for hairpin substrates and MRX facilitates Sae2 endonuclease activity on single-stranded DNA adjacent to a hairpin through its 3' to 5' exonuclease activity.

However, unlike Mre11 or Rad50, Sae2 is poorly conserved among eukaryotes. Even among yeast species, only regions in the C-terminus of the protein show significant conservation. Sae2 from budding yeast, or the Sae2 ortholog CtIP from human, fission yeast and worms are so different from each other that they only share a small RHR motif and FPSTQ region close to the C- terminus of the protein (Figure 4.1), which is required

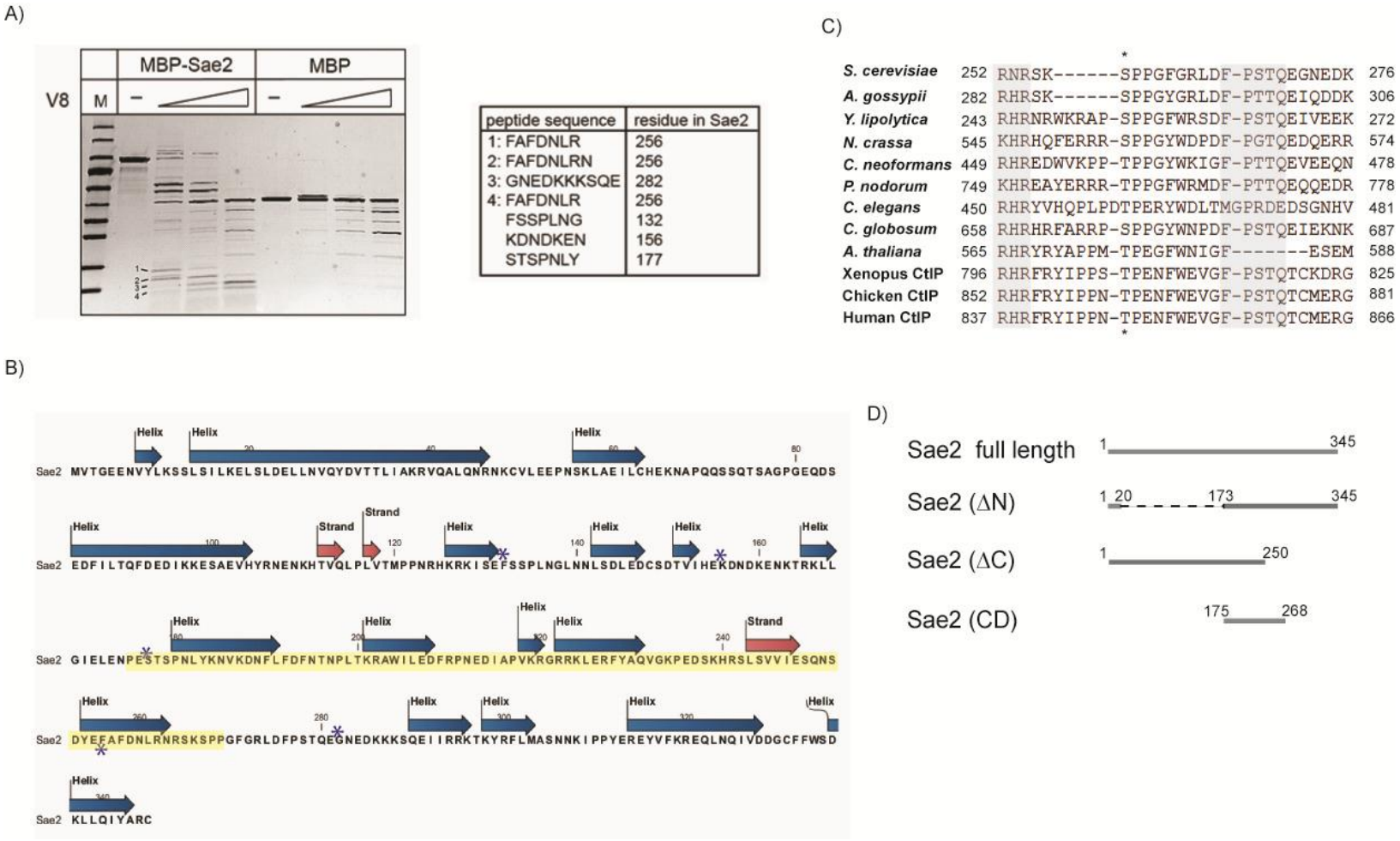


Figure 4.1 Partial proteolysis of recombinant Sae2.

A) Partial proteolysis was performed for recombinant MBP-Sae2 with V8 protease. MBP protein alone was also digested as a control. Proteolysis products were resolved in an 8% SDS-PAGE gel. Four bands unique to MBP-Sae2 were identified, and subjected to N-terminal sequencing analysis. B) Predicted secondary structure of Sae2. The acidic residues identified by N-terminal sequencing after V8 partial proteolysis are highlighted with a star. The Sae2 central domain is highlighted in yellow. C) A sequence alignment of RHR and FPSTQ motifs in Sae2/CtIP/Ctp1/Com1 from *S. cerevisiae*, *A. gossypii*, *Y. lipolytica*, *N. crassa*, *C. neoformans*, *P. nodorum*, *C. elegans*, *C. globosum*, *A. thaliana*, xenopus, chicken, and human. "*" indicates S/T sites phosphorylated by CDK. D) Schematic representation of the Sae2 full length protein, Sae2 (ΔN), Sae2 (ΔC) and Sae2 (CD) deletion mutants. The phenotypes of Sae2 (ΔN) and Sae2 (ΔC) were illustrated by Lengfeld et al. The Sae2 (CD) mutant is analyzed in this study.

for CDK phosphorylation (Huertas, Cortes-Ledesma et al. 2008).

CHARACTERIZATION OF A NOVEL ENDONUCLEASE DOMAIN

Except for the RHR motif important for CDK phosphorylation close to the C-terminus of the protein, Sae2 has no obvious functional motifs or domains. Lengsfeld et al. characterized some deletion mutants of Sae2 (*sae2 (ΔN)* and *sae2 (ΔC)*) that helped to define the functions of the N- and C- terminal domains (Lengsfeld, Rattray et al. 2007). The Sae2 (ΔN) mutant (deletion of residues 21 - 172) was deficient in DNA binding, while the Sae2 (ΔC) mutant (deletion of residues 251 - 345) showed deficiency in cooperative nuclease activity with MRX, but still showed residual endonuclease activity.

To determine more accurately where the domain boundaries are located in Sae2, I performed V8 partial proteolysis on the recombinant purified MBP-Sae2 protein (Figure 4.1 A). Four bands unique to MBP-Sae2 proteolysis were analyzed by N-terminal sequencing (Edman degradation, ICMB core facility). This revealed several acidic residues sensitive to V8 digestion (Figure 4.1B). Taking the partial proteolysis results and the predicted secondary structure into consideration, I constructed an expression vector for the central domain of Sae2 (175aa ~ 268aa). I expressed and purified this central domain of Sae2 (Sae2 (CD)) as I did for recombinant wild-type Sae2. Surprisingly, this domain alone shows a similar level of endonuclease activity toward 5' [^{32}P]-labeled flap DNA as compared to wild-type Sae2. However, the cleavage pattern is slightly different between wild-type Sae2 and the recombinant central domain. While wild-type Sae2 has a strong preference towards the single/double stranded DNA junction, which leads to one of the major products of about 17 nt after endonuclease cleavage, the purified central domain of Sae2 did not yield this product.

To characterize this central domain further, I mutated many conserved residues

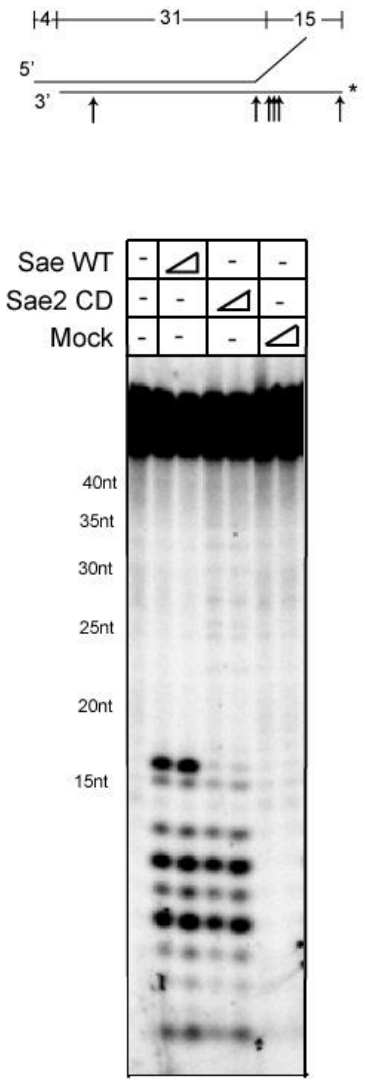


Figure 4.2 Recombinant Sae2 Central Domain (a.a. 175 to 268) has endonuclease activity.

5nM and 10nM of WT (wild-type), CD (Central Domain), and an equal volume of Mock fraction (same fraction of a mock purification not expressing recombinant Sae2 but applied to all Sae2 purification steps) was used in the endonuclease assay. Products of endonuclease assay were analyzed in a 20% denaturing polyacrylamide gel.

within this domain to express recombinant mutant forms of His-MBP-Sae2. I mostly targeted acidic residues because many nucleases use acidic residues to coordinate metal ions that are essential for catalysis. I expressed and purified these Sae2 mutants from *E. coli*, and tested their endonuclease activity *in vitro* using either 5' [^{32}P]-labeled oligonucleotides with a flap at one end or 5' [^{32}P]-labeled oligonucleotides with a hairpin at the end. The DNA binding ability of Sae2 mutants was tested in a gel mobility shift assay using an internally labeled 249 bp double-stranded DNA as a substrate. The protein stability of Sae2 mutants *in vivo* was analyzed by western blot in TCA extracts of yeast cells expressing Sae2 mutants. Some mutants showed interesting phenotypes *in vitro* and were further analyzed *in vivo* by measuring the rate of processing of hairpin intermediates in ALE94 strains (gift from K. Lobachev) expressing Sae2 mutants. In ALE94 (Lobachev, Gordenin et al. 2002), 320bp Alu inverted repeats are present in the *Lys2* gene on the Chromosome II in an inverted orientation. Inverted repeats can form a cruciform structure *in vivo*, which is a vulnerable structure. A cruciform can be cleaved by Mus81 or other enzymes, in an MRX and Sae2-independent manner (Cote and Lewis 2008). In wild-type cells, MRX and Sae2 remove the hairpin structure and prepare DNA ends for long range resection and homologous recombination, which yield wild-type *LYS2* gene. In *sae2* mutant cells, stable hairpin structures lead to DNA replication of the hairpin and formation of large palindromes in the chromosome. I summarized the phenotypes of Sae2 mutants known so far in Table 4.1.

The central domain contains a conserved region from D193 to N212. Disruption of any of these following conserved sites (D193A, N197I, P198L, and W204L) causes protein instability. I could not detect Sae2 protein in cell lysates from yeast cells expressing any of the above mutants from a plasmid under the control of *SAE2* native

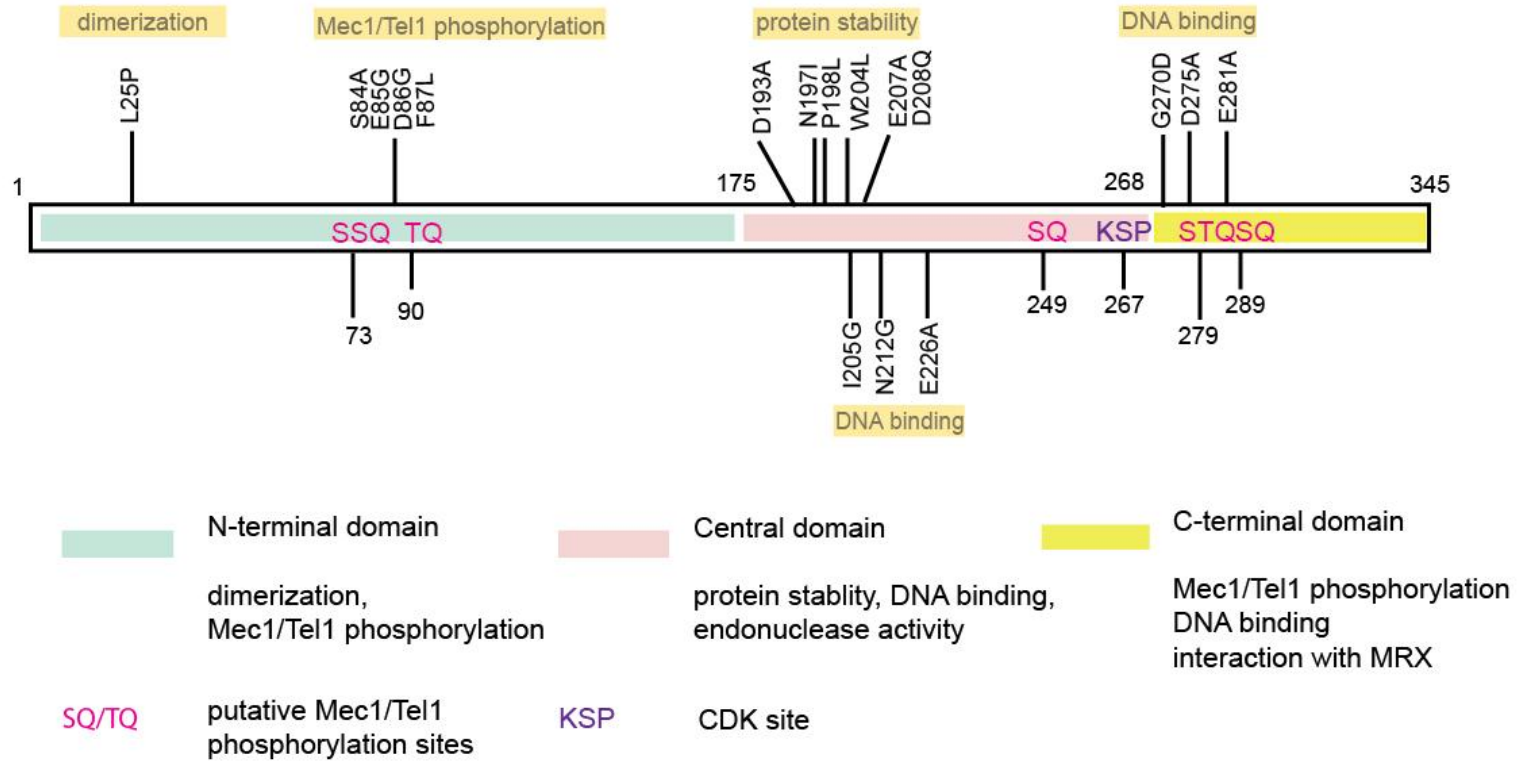


Figure 4.3 Schematic summary of the phenotype of Sae2 mutants.

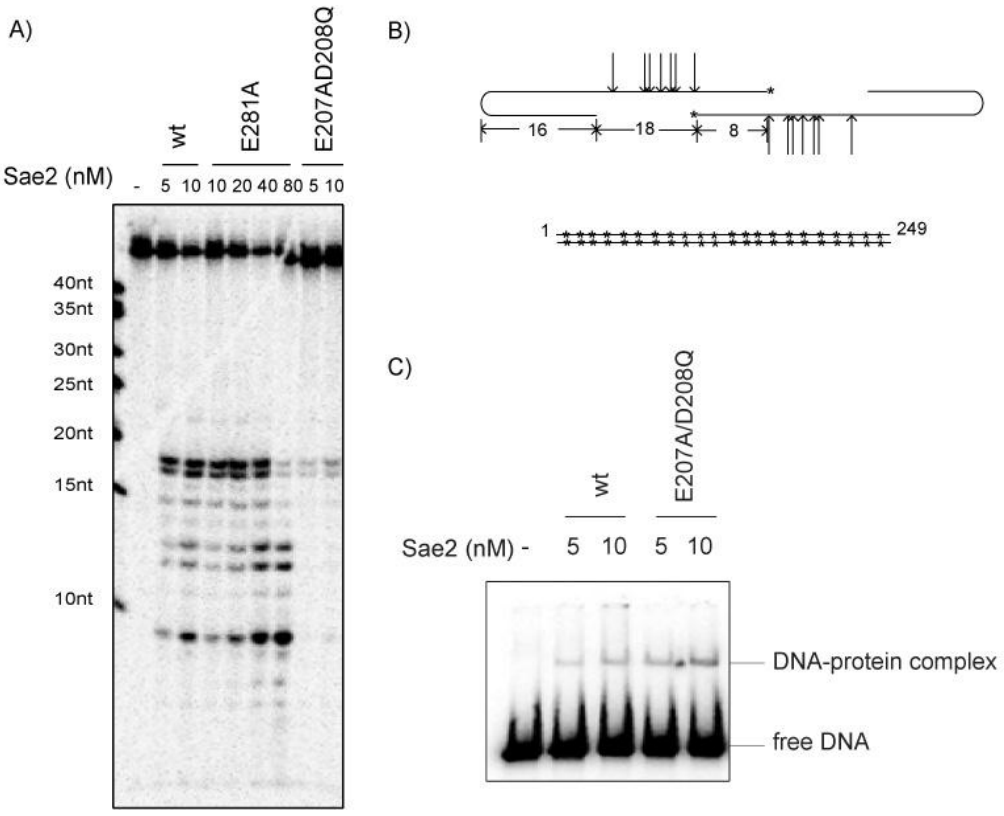


Figure 4.4 Nuclease-deficient Sae2 mutant

5nM and 10nM of wild-type Sae2, and Sae2 (E207A/D208Q) was used in the A) endonuclease assay and B) Schematic representation of the substrate used in Sae2 endonuclease assay (top panel) and gel mobility shift assay (bottom panel). Cleavage sites are shown with arrows. C) gel mobility shift assay. Products of endonuclease assay or DNA binding assay were analyzed in a 20% denaturing polyacrylamide gel or 6% native polyacrylamide gel, respectively.

promoter (Table 4.1). This stretch of conserved residues contains two more acidic residues, E207 and D208. When I mutated E207 to alanine, I found that the recombinant Sae2 (E207A) mutant has a similar level of endonuclease activity as the wild-type protein (data not shown). I also made a construct to express Sae2 (E207A/D208A) with mutations in both residues. Surprisingly, I could not get a good yield of this mutant protein from *E. coli*. Since mutation of D208 to alanine caused this expression problem in *E. coli*, I mutated D208 to different residues. Taking the similarity of amino acids into consideration, I mutated D208 to glutamine, asparagine, and aspartate. Sae2 (E207A/D208Q), Sae2 (E207A/D208N) and Sae2 (E207A/D208E) could all be expressed and purified normally from *E. coli*, although the yields of protein were significantly less than wild-type Sae2. The activity of Sae2 (E207A/D208E) was similar to wild-type (data not shown). In contrast, Sae2 (E207A/D208Q) had only 20% activity compared to wild-type Sae2, although it bound DNA normally (Figure 4.3). This mutant was a good candidate for a nuclease-deficient Sae2 mutant, however, when I further analyzed this Sae2 (E207A/D208Q) mutant *in vivo*, TCA extraction results indicated that this mutant also failed to express well in yeast (Table 4.1). I also expressed and purified Sae2 (D208Q), the endonuclease activity of this single mutant was similar to that of wild-type Sae2.

Residues immediately following the a.a. 193 – 208 region (I205, N212, E226) contribute to the DNA binding ability of Sae2. The expression level of I205G, N212G, and E226A mutants is normal in yeast cells, but purified recombinant protein binds relatively poorly to DNA substrates *in vitro*, compared to wild-type Sae2. All mutants in this category also showed a significant decrease in the rate of hairpin intermediate processing (Table 4.1).

EFFECT OF SAE2 MUTATIONS IN THE N-TERMINUS AND C-TERMINUS

Lengsfeld et al. reported that the Sae2 ΔC mutant (deletion of residues 251–345) showed impaired functional cooperativity with MRX, but still exhibited residual endonuclease activity. At the C-terminus of Sae2, they also identified Sae2 (G270D) as a mutant deficient in DNA binding.

I found that two more residues close to G270, D275 and E281, are also essential for the association of Sae2 with DNA (Table 4.1).

The C-terminus of Sae2 contains two putative Mec1/Tel1 phosphorylation sites (T279, S289). The RHR motif, conserved among many eukaryote species, is followed by CDK phosphorylation site S267, and another conserved FPSTQ region. Although the C-terminus of Sae2 is one of the most conserved regions, no residues have been identified so far to contribute significantly to Sae2 endonuclease activity.

The N-terminus of Sae2 is the least conserved part of the entire protein. Previous data from Lengsfeld et al. indicated recombinant wild-type Sae2 protein has a consistent distribution of multimer, dimer, and monomer, observed after the protein was analyzed by gel filtration on a Superdex 200 column, while the Sae2 ΔN mutant (deletion of residues 21–172) failed to dimerize. Sae2 (ΔN) was also deficient in DNA binding. Using random mutagenesis, Kim et al. also identified the Sae2 (L25P) mutant in the N-terminus that blocks Sae2 multimerization. Sae2 (L25P) was demonstrated to be similar to Δ*sae2* in all *in vivo* analysis (Kim, Vijayakumar et al. 2008), including sensitivity to MMS, CPT, synthetic lethality with Δ*rad27*, and sporulation efficiency. By constructing and expressing two deletion mutants from the N-terminus (ΔN120 and ΔN170), they identified another domain required for dimerization between a.a. 120 and a.a. 170.

When I purified recombinant Sae2 (L25P) protein from *E. coli*, it was unusual

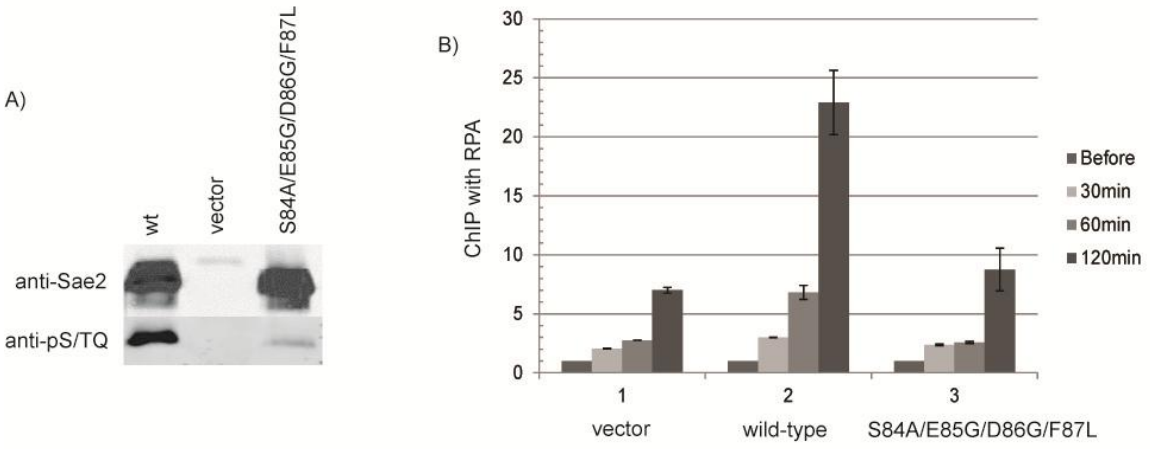


Figure 4.5 Sae2 (S84A/E85G/D86G/F87L) is deficient in Mec1/Tel1 phosphorylation and resection upon DNA damage.

A) Δ*sae2* strains carrying plasmids expressing a vector control, Flag-tagged Sae2 wild type or S84A/E85G/D86G/F87L mutant were treated with 0.03% MMS for 4 hours. Sae2 was immunoprecipitated with anti-Flag agarose beads, and western blot was performed using either anti-Sae2 or anti-pS/TQ antibodies. B) ChIP analysis was performed using an anti-RPA antibody in Δ*sae2* strains carrying plasmids expressing a vector control, wild type, or S84A/E85G/D86G/F87L of Sae2. Quantification of PCR signals from a set of primers that anneal 0.2 kb from an HO break at different durations of HO expression are shown (values are mean ± s.d.) (by Sang Eun Lee, UTHSCSA).

that Sae2 (L25P) did not exhibit the multimeric or dimeric forms after gel filtration. However, Sae2 (L25P) behaved like wild-type in endonuclease assay *in vitro*, though it was defective in repairing hairpin structure *in vivo* (Lys2 conversion assay) (Table 4.1). It is not clear why the Sae2 (L25P) mutant exhibits this phenotype *in vivo*, yet appears to be functional *in vitro*. Another interesting mutant I found in the N-terminus is the quadruple mutant S84A/E85G/D86G/F87L. It shows only a very slight decrease in endonuclease activity, and it is about six fold less efficient in processing hairpin intermediates. When analyzed in a Chromatin Immunoprecipitation (ChIP) assay to identify the extent of resection after DSBs, a Δ*sae2* strain expressing Sae2 (S84A/E85G/D86G/F87L) was almost as deficient as a Δ*sae2* strain (Figure 4.5 B).

Sae2 is known to be phosphorylated by Mec1/Tel1 after DNA damage (Baroni, Viscardi et al. 2004). To test whether these N-terminal mutations might affect Sae2 phosphorylation, I expressed Flag-tagged wild type or (S84A/E85G/D86G/F87L) Sae2 in a Δ*sae2* strain, and immunoprecipitated Sae2 with anti-Flag agarose beads. In the absence of MMS treatment, neither wild type Sae2 nor Sae2 (S84A/E85G/D86G/F87L) showed any significant signals when blotted with anti-phospho-S/TQ antibody. With MMS treatment for 4 hours at the concentration of 0.03%, as shown in Figure 4.5 A, wild-type Sae2 was strongly phosphorylated, while the phosphorylation level of Sae2 (S84A/E85G/D86G/F87L) was much weaker. This result indicates that the stretch of amino acids from S84 to F87 could be important residues interacting with Mec1/Tel1, or recognized by these two kinases upon DNA damage, and that Mec1/Tel1 phosphorylation of the N-terminus of Sae2 is important for Sae2 function in DSB end processing.

CONCLUSIONS

		dimerization in vitro	protein stability in yeast	DNA binding in vitro	endonuclease activity in vitro	processing of hairpin intermediates in vivo (median frequency ±SDX10-7)
wt		Yes	+++	+++	+++	543±173
null		N/A	N/A	N/A	N/A	6±2
ΔN		No	+++	-	-	6±1
ΔC175		Yes	+	-	+	13±3
ΔC268		Yes	+++	-	+	13±4
CD		No	+	+++	(+++)	12±2
D193A		Yes	-	N/A	N/A	ND
N197I		Yes	-	N/A	N/A	ND
P198L		Yes	-	N/A	N/A	ND
W204L		Yes	-	N/A	N/A	ND
E207A/D208Q		Yes	-	+++	+	ND
I205G		Yes	+++	+	+	26±10
N212G		Yes	+++	++	++	71±16
E226A		Yes	+++	++	++	122±78
G270D		Yes	+++	-	-	N/A
D275A		Yes	+++	-	+	N/A
E281A		Yes	+++	+	+	N/A
L25P		No	+++	+++	+++	8±2
S84A/E85G/ D86G/F87L		Yes	+++	+++	++	89±20

Table 4.1 Summary of the phenotypes of Sae2 mutants.

Recombinant wild type and different mutant forms of MBP-Sae2 were purified from *E. coli*. Endonuclease activity *in vitro* was tested using either 5' [^{32}P]-labeled flap DNA or 5' [^{32}P]-labeled hairpin DNA. The DNA binding ability of Sae2 protein *in vitro* was tested in gel mobility shift assays using internally [^{32}P]-labeled 249 bp double-stranded DNA as substrates. The protein stability of Sae2 mutants was analyzed by western blot of TCA extracts of Δ*sae2* strains carrying low copy plasmids expressing wild type or mutant forms of Sae2 under the control of the *SAE2* promoter. The rate of processing of hairpin intermediates were measured in Δ*sae2* ALE94 strains (gift from K.Lobachev) carrying plasmids expressing wild type or mutants of Sae2. The rate of recombination was calculated for each strain by fluctuation analysis (Spell and Jinks-Robertson 2004) and the standard deviation is as shown. "+++" indicates phenotypes similar to wild type Sae2. "(+++)" indicates a level of endonuclease activity similar to that of wild type Sae2, but with different pattern of cleavage products. "-" indicates phenotypes similar to null. "+" and "++" are intermediate phenotypes between "+++" and "-". Schematic representations of each mutant are as illustrated. N-terminal domain is in blue, central domain in red, and C-terminal domain in yellow. Point mutation sites are marked with "X" at corresponding positions.

Domain structure

Yeast Sae2 was demonstrated to be an endonuclease *in vitro*, however, it was lacking any obvious nuclease domain. CtIP, the functional ortholog of Sae2 in mammals, is significantly larger than Sae2 (897 a.a. v. 345 a.a.). Similar to Sae2, CtIP is also lacking any recognizable domains except for a coiled-coiled region at the N-terminus, which was known to mediate homodimerization (Dubin, Stokes et al. 2004). The N- terminus of Sae2 is barely conserved even among fungi species, while its C-terminus shares several conserved residues with its functional orthologs in fission yeast, worms and human cells near the CDK phosphorylation site (Huertas, Cortes-Ledesma et al. 2008). A previous study of Sae2 illustrated that the N- terminus of Sae2 is important for Sae2 dimerization as well (Lengsfeld, Rattray et al. 2007; Kim, Vijayakumar et al. 2008). The region from a.a. 120 to 170 was shown to be essential for Sae2 dimerization. *In vitro and in vivo* analysis indicated that Sae2 ΔC mutant (deletion of a.a. 251 to 345) was partially deficient in endonuclease activity, as well as in processing DSB ends, very likely due to its much weaker interaction with MRX (Lengsfeld, Rattray et al. 2007; Nicolette, Lee et al. 2010).

I performed partial proteolysis on recombinant Sae2 protein, and identified a central domain (a.a. 175 to 268). Considering this structural evidence, predicted secondary structure, and sequence conservation of Sae2, I hypothesized that Sae2 has three domains, N-terminal (a.a. 1 to 174), Central (a.a. 175 to 268) and C-terminal (a.a. 269 to 345). I generated point mutants in each domain using site-directed mutagenesis, and studied their phenotypes *in vivo* and *in vitro*, as summarized in Table 4.1 and Figure 4.3. These results further support the boundaries between these three domains I hypothesized.

Function of the N-terminal and C-terminal domains

The N-terminal domain (a.a. 1 to 174) clearly plays a regulatory role in Sae2 activity through homodimerization and also post-translational modification. Lengsfeld et al. demonstrated that recombinant Sae2 lacking a.a. 21 to 173 could not homodimerize or bind DNA as wild type Sae2 did *in vitro*. I demonstrated that recombinant Sae2 (L25P) did not appear in a homodimer form after purification. Similar results were shown in previous study using Sae2 (L25P) immunoprecipitated from yeast cells. Unexpectedly, Sae2 (L25P) shows normal endonuclease activity *in vitro*, but Δ*sae2* strains carrying plasmids expressing Sae2 (L25P) were completely deficient in processing hairpin intermediates in yeast. The opposite phenotypes of Sae2 *in vivo* and *in vitro* indicated that instead of directly regulating Sae2 activity, the dimerization of Sae2 may regulate its availability to DSBs, or its dynamics with other proteins responsible for DSB repair, such as MRX.

Another mutant I identified at the N-terminus is the S84A/E85G/D86G/F87L quadruple mutant. It showed phenotypes similar to *sae2* null strains in a resection assay measured by ChIP with RPA. In response to DNA damage, Sae2 (S84A/E85G/D86G/F87L) was not phosphorylated efficiently by Mec1/Tel1 compared to wild-type Sae2. There are two putative Mec1/Tel1 phosphorylation sites at the N-terminus of Sae2: S73 and T90. The mutation site of this quadruple mutant is close to both Mec1/Tel1 phosphorylation sites. It is possible that Sae2 (S84A/E85G/D86G/F87L) cannot interact well with Mec1/Tel1, so that it is not phosphorylated at the N-terminus, and fails to act properly in DSB resection upon DNA damage.

The C-terminus of Sae2 (a.a. 269 to 345) is the one of the most conserved parts of this protein, and undergoes several kinds of post-translational modification. The RHR

motif is close to the C-terminus of Sae2, which was observed to be important for Sae2 CDK phosphorylation at S267 (Huertas, Cortes-Ledesma et al. 2008). And RHR motif was identified in CtIP/ctp1 in many higher eukaryotes. T279, and S289 are also putative Mec1/Tel1 phosphorylation sites (Baroni, Viscardi et al. 2004) located close to S267. A previous study identified a DNA-binding deficient mutant, G270D, in this region (Lengsfeld, Rattray et al. 2007). I identified two more mutants: D275A and E281A, in Sae2 that exhibit deficiency in binding to DNA. Deletion mutant of Sae2 lacking this C-terminal domain exhibited a residual level of endonuclease activity *in vitro*, but was barely able to process hairpin intermediates *in vivo*. Previous studies also showed that Sae2 ΔC (deletion of a.a. 251 to 345) fails to process hairpin DNA *in vitro* in the presence of MRX (Lengsfeld, Rattray et al. 2007). Thus, the C-terminal domain of Sae2 is responsible for promoting DNA binding, post-translational modifications, and cooperating with MRX to processing hairpin ends. The latter two are very likely to be related, and contribute to the regulation of Sae2 activity in S and G_2 phase upon DNA damage.

Location of the nuclease motif

The recombinant central domain (a.a.175 - 268) of Sae2 showed endonuclease activity *in vitro* (Figure 4.2). Although its cleavage pattern was slightly different from that of wild-type, Sae2 central domain clearly bound DNA and processed the DNA at the single/double-stranded DNA junction as well as single-stranded DNA close to 5'end in a 5' labeled flap DNA. However, most mutants identified so far between a.a. 197 and a.a. 208 disrupted the protein stability of Sae2 in yeast. And I had difficulty in purifying most of them in recombinant form from *E. coli*. One of the mutants I purified successfully but in low yield from *E. coli* is Sae2 (E207A/D208Q); it bound DNA similar to the wild-type

protein, but showed only 20% endonuclease activity when compared to that of wild-type. In this central domain, I also identified other mutants (I205G, N212G, E226A) that were weak in DNA-binding *in vitro*, which very likely results in their partial deficiency in processing hairpin intermediates *in vivo.*

Role of different multimeric forms

Recombinant Sae2 appears in three forms: multimer, dimer, and monomer, after gel filtration chromatography. These three forms have been verified using sedimentation analysis using analytical ultracentrifugation (Lengsfeld, Rattray et al. 2007). All the *in vitro* analysis I did in my study was performed using monomer form of Sae2, which shows the most activity *in vitro*. Sae2 (L25P) as well as Sae2 lacking N-terminal domain did not fail to form dimers as wild-type did *in vitro*. Immunoprecipitation from yeast cells expressing Sae2 (L25P) and various deletion mutants from the N-terminus of Sae2 indicated that there are two domains in the N-terminus responsible for homodimerization, one at or around L25, and the other between a.a. 120 -170 (Kim, Vijayakumar et al. 2008). However, little was known about the transition between different multimeric forms of Sae2 *in vivo*, and the physiological importance of the transition between different multimeric forms is under study.

CHAPTER 5 THE EFFECT OF SAE2 PHOSPHORYLATION ON EXO1-MEDIATED DSB RESECTION

INTRODUCTION

The Ku heterodimer has been shown by many groups to antagonize DSB processing. Deletion of yKu was found to increase the initiation of resection both at DSB ends and telomeres (Lee, Moore et al. 1998; Maringele and Lydall 2002; Clerici, Mantiero et al. 2008). Overexpression of Ku also significantly delays resection in G_2 phase (Clerici, Mantiero et al. 2008). *In vitro* analysis using recombinant human Ku and MRN complexes, and yeast Ku and MRX complexes, both indicated that Ku inhibits the association between MRX(N) and DNA (Paull and Gellert 1999; Shim, Chung et al. 2010). Recent studies showed that it is likely that there is a competition between MRX and Ku for DSB ends. The absence of *MRE11* or *RAD50* induced an approximately 20-fold increase of yKu binding at DSB sites in yeast (Shim, Chung et al. 2010). Deletion of yKu significantly suppressed the radiation sensitivity of a Δ*mre11* mutant strain, but did not fully restore it to the wild-type level. Deletion of *EXO1* reverted the suppression by ΔyKu, and overexpression of *EXO1* increased the radiation resistance of Δ*mre11* (Mimitou and Symington 2010). These results suggest that in the absence of MRX, Ku blocks access of Exo1 to DSB ends. However, since deletion of yKu cannot recover the IR resistance of the Δ*mre11* mutant to wild-type level, MRX plays an additional role in DNA end resection other than competing with yKu.

With respect to DSB resection, Δ*sae2* and Mre11 nuclease-deficient (*mre11-nd*) strains exhibit a similar phenotype. They both accumulated large palindromic duplication products due to improper processing of hairpin ends (Rattray, McGill et al. 2001; Lobachev, Gordenin et al. 2002; Clerici, Mantiero et al. 2005). The effect of *SAE2*

deletion or expression of *mre11-nd* mutant toward yKu accumulation at DSB ends was both moderate. The level of yKu protein increased only about 2.5-fold in a Δ*sae2* strain or a *mre11-nd* strain compared to a wild-type yeast strain (Shim, Chung et al. 2010). However, the Δ*sae2* strain was more sensitive to IR than the *mre11-nd* strain. Deletion of yKu fully recovered the IR resistance of the Δ*sae2* strain, while it only increased the IR resistance of the *mre11-nd* strain at 800 Gy by seven-fold. This suppression effect is dependent on both *EXO1* and *SGS1* (Mimitou and Symington 2010). Thus, in the absence of Sae2 or the presence of a structurally intact but functionally compromised MRX complex, yKu inhibits the function of Exo1 and Sgs1 by accumulating at the DSB ends.

These results indicate that MRX and Sae2 neutralize the inhibitory effect of Ku towards Exo1, most likely by removing Ku from DNA ends. This hypothesis was further supported *in vitro* with recombinant Ku, Exo1, MRX and Sae2 protein (Shim, Chung et al. 2010). Exo1 processes 5' ends competently, but the activity is fully suppressed in the presence of Ku. The addition of MRX and Sae2 partially recovers Exo1-mediated resection under the same reaction conditions.

CDK and Mec1/Tel1 phosphorylation of Sae2 has long been suggested to be correlated with DSB ends resection initiation and commitment to HR (Baroni, Viscardi et al. 2004; Huertas, Cortes-Ledesma et al. 2008). Yeast strains expressing a Sae2 (5A) mutant, in which all five putative Mec1/Tel1 phosphorylation (S/T)Q sites (S73, T90, S249, T279, and S289) were mutated to alanine, showed increased sensitivity to MMS. Sae2 is known to be phosphorylated by CDK at Ser267 during S and G_2 phase. Phenotypes similar to Δ*sae2* strains were observed in yeast cells expressing the Sae2 (S267A) mutant, including hypersensitivity to DNA-damaging reagents, deficiency of meiotic recombination, reduced DNA end resection, and reduced hairpin-induced

homologous recombination. In contrast, cells expressing Sae2 (S267E) which mimics constitutive CDK phosphorylation, have phenotypes similar to those of wild-type. Little detail has been demonstrated so far about the effect of these modifications on the function of Sae2 or on its dynamics with MRX, or Ku. To characterize the effect of these modifications, I purified recombinant MBP-Sae2 mimicking phosphorylation by CDK and Tel1/Mec1, and investigated their roles in DNA ends resection *in vitro*.

SAE2 PHOSPHORYLATION HAS NO EFFECT ON ITS ENDONUCLEASE ACTIVITY

Sae2 was shown to be an endonuclease *in vitro*, and it has a preference for single-stranded DNA and single-stranded/double-stranded DNA junctions (Lengsfeld, Rattray et al. 2007). It is possible that modification of Sae2 by CDK and/or Mec1/Tel1 may enhance its endonuclease activity in G_2 phase upon DNA damage, so as to regulate the choice between NHEJ and HR. Using an *E. coli* expression system, I purified recombinant Sae2 (S267A) to mimic the non-phosphorylatable form of CDK phosphorylation, Sae2 (S267E) to mimic CDK phosphorylation, and Sae2 (5D/S267E) to mimic CDK and Mec1/Tel1 phosphorylation. The Sae2 (5D) and Sae2 (5A) mutant were purified and characterized previously (Lengsfeld, Rattray et al. 2007). Sae2 (5D) bound DNA normally and was shown to have endonuclease activity similar to wild-type Sae2. Sae2 (5A) was shown to compromise the DNA-binding and endonuclease activity of Sae2, even though recombinant wild-type Sae2 purified from *E. coli* is not phosphorylated. Thus the Sae2 (5A) mutations may cause other functional deficiencies that are unrelated to its phosphorylation.

As shown in Figure 5.1, the Sae2 (S267E) and Sae2 (5D/S267E) mutants are similar to wild-type Sae2 in endonuclease activity. Unexpectedly, Sae2 (5D/S267E) exhibited even about two-fold decrease in endonuclease activity compared that of

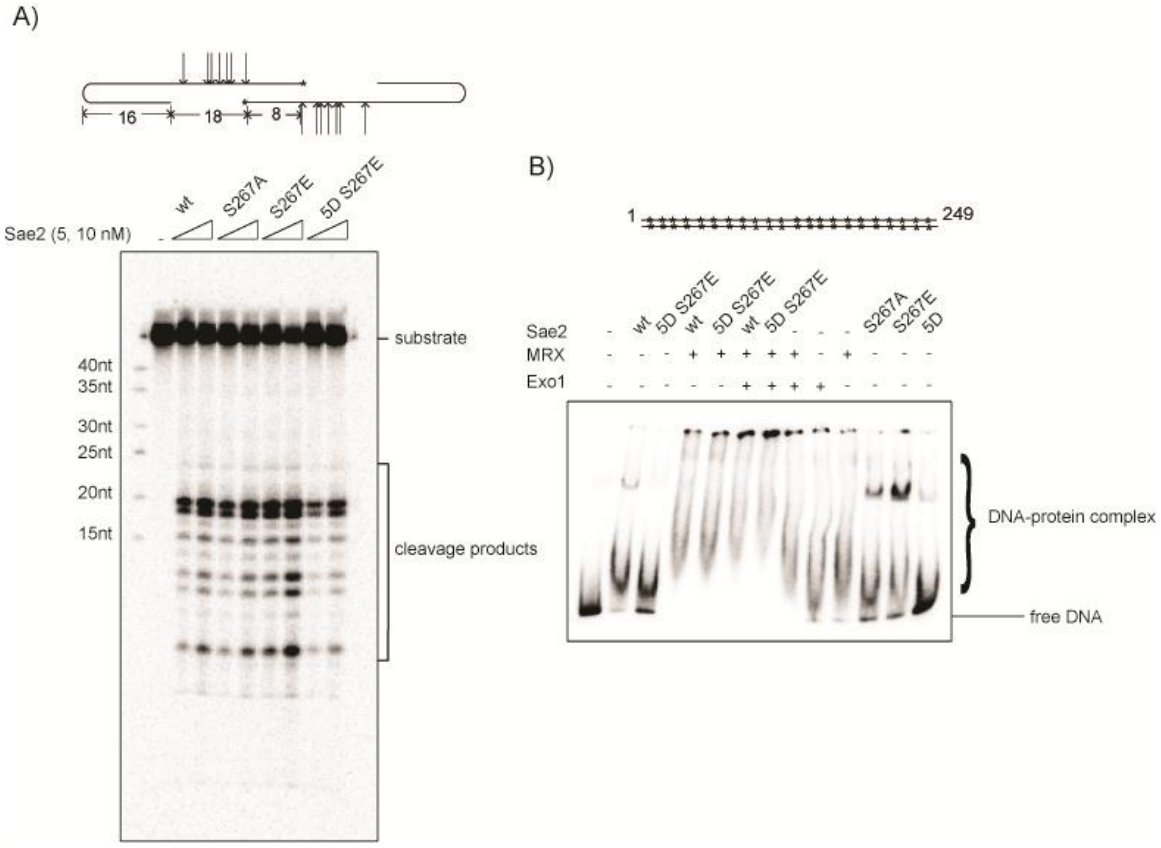


Figure 5.1 Endonuclease activity and DNA binding ability of Sae2 (S267A), Sae2 (S267E), and Sae2 (5D/S267E).

2.5 nM and 5 nM MBP-Sae2 protein was used in an endonuclease assay with a 5'-labeled hairpin substrate as shown (A). The products of the endonuclease assay were resolved in a 20% denaturing polyacrylamide gel. Sae2 cleaves the hairpin substrate at several sites in the single-stranded region adjacent to the hairpin stem (arrows in the diagram). (B) DNA binding by Sae2 with recombinant MRX complex and Exo1 was measured using a gel mobility shift assay. MRX and Sae2 form a protein-DNA complex that migrates higher in the gel compared to the complexes formed with either complex separately. And this is further shifted into the well with the addition of Exo1. The products of the

DNA binding assay were resolved in a 6% native polyacrylamide gel and analyzed by phosphorimager.

wild-type Sae2, most likely due to its weaker association with DNA. Thus, the endonuclease activity of these Sae2 mutants *in vitro* does not seem to correlate with their ability to initiate resection *in vivo*.

MRX and Sae2 were also demonstrated to enhance Exo1 5' endonuclease and exonuclease activity *in vitro* (Nicolette, Lee et al. 2010). To test whether Sae2 (S267A), Sae2 (S267E), and Sae2 (5D/S267E) play a distinctly different role in assisting DNA ends resection mediated by Exo1, I performed the *in vitro* DNA end resection assay using a linearized plasmid as a substrate. The resection was further analyzed by non-denaturing southern blot using an RNA probe complementary to 3′ strand of the linearized pNO1 substrate in a 1 kb region adjacent to the *Sph*I site (Figure 5.2). Although Sae2 (5D/S267E) did not dramatically increase the resection efficiency of Exo1 by itself, wild-type Sae2 and Sae2 (5D/S267E) stimulate Exo1 exonuclease activity to a similar level in the presence of MRX. However, Sae2 (5D/S267E) did not show any increase in stimulatory activity in comparison to wild-type Sae2.

SAE2 PHOSPHORYLATION ENHANCES ITS ABILITY TO REVERSE THE INHIBITORY EFFECT OF KU ON EXO1

The interplay between Ku and MRX/Sae2 has been well demonstrated *in vivo and in vitro* (Mimitou and Symington 2010; Shim, Chung et al. 2010). Previous results suggested that MRX/Sae2 may release the inhibition of Ku towards Exo1 by directly competing with Ku at DSB ends during S and G_2 phase. This event is critical for yeast cells to commit to HR instead of NHEJ during S and G_2 phases of the cell cycle when a second copy of the chromosomes is present. CDK has been shown to be required for the dramatic increase in resection efficiency that occurs in S and G_2 phases of the cell cycles (Huertas, Cortes-Ledesma et al. 2008; Kosugi, Hasebe et al. 2009). So far, only two

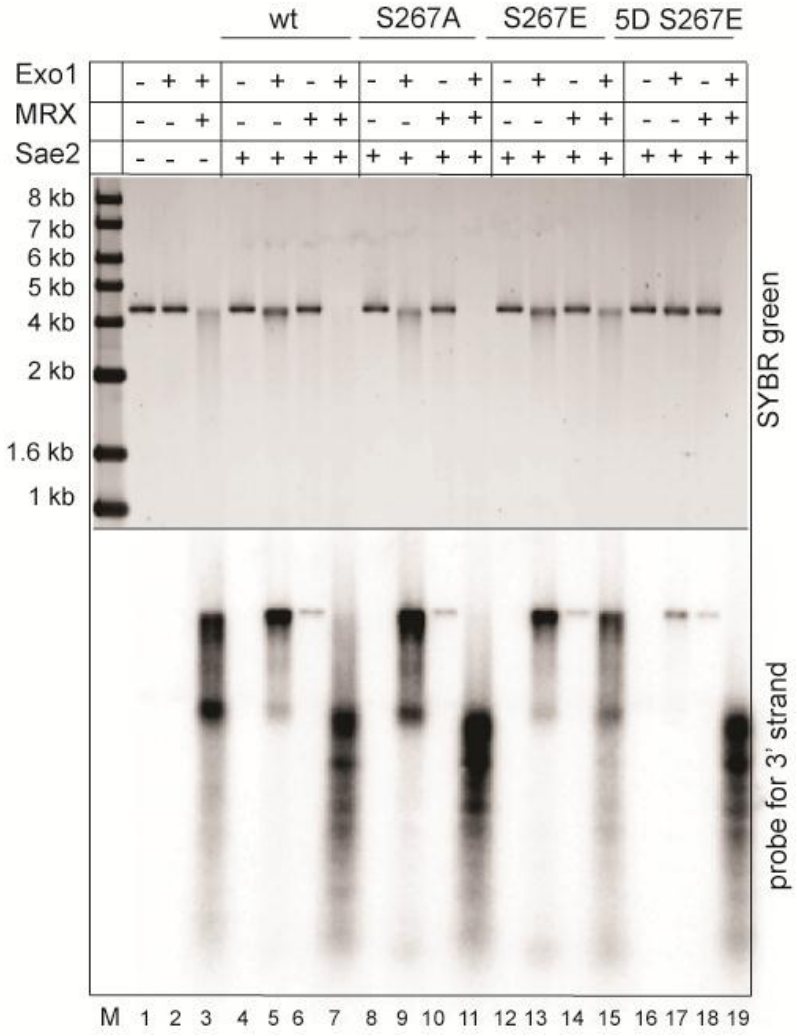


Figure 5.2 Exo1 exonuclease activity stimulated by Sae2 mutants.

Exo1 (0.1 nM), MRX (10 nM), and Sae2 (2.5 or 5 nM) were tested in the resection assay with 4.5 kb pNO1 plasmid linearized with *Sph*I-HF as indicated above. Exonuclease products were resolved in a 1% agarose gel, and stained with SYBR green (top panel). A non-denaturing southern blot was performed after SYBR green staining, and probed with an RNA complementary to 3′ strand of the linearized pNO1 substrate in a 1 kb region adjacent to the *Sph*I site (bottom panel).

targets in resection are known for CDK regulation. Sae2 is directly phosphorylated by CDK at S267, and the nuclear entry of Dna2 is also regulated by CDK (Huertas, Cortes-Ledesma et al. 2008; Kosugi, Hasebe et al. 2009). Yeast cells expressing Sae2 (S267A) mutant are hypersensitive to DNA-damaging reagents, deficient in meiotic recombination, unable to process DSB ends, and less efficient in hairpin-induced homologous recombination.

By using recombinant protein complexes *in vitro*, I found that the inhibition of Ku towards Exo1 is relieved most efficiently with the combined effect of MRX and Sae2 (Figure 5.3). MRX alone only partially alleviates the inhibition by Ku. I further tested the effect of different Sae2 mutants (S267E, 5D/S267E) on the release of Ku inhibition. By limiting the amount of MRX in the assay, I show here in Figure 5.4 and Figure 5.5 that there is a significant difference between wild-type Sae2, Sae2 (S267E), and Sae2 (5D/S267E) mutants with respect to stimulating Exo1 exonuclease activity in the presence of Ku. The 5D/S267E mutant and S267E mutant show a more robust ability to promote Exo1 activity in the presence of Ku. The stimulation was further quantified by quantitative PCR using two sets of primers located 29nt and 1025nt, respectively, from the 3' ends of linearized pNO1 substrate. Together with MRX, Sae2 (5D/S267E) was much more proficient than wild-type Sae2 in stimulating Exo1- mediated resection, particularly long-range resection, and alleviating the inhibitory effect of Ku.

CONCLUSIONS

Effect of phosphorylation on Exo1 activity

Sae2 is the phosphorylation target of both CDK during S/G_2 phase and Mec1/Tel upon DNA damage. Genetic evidence indicated that CDK phosphorylation and

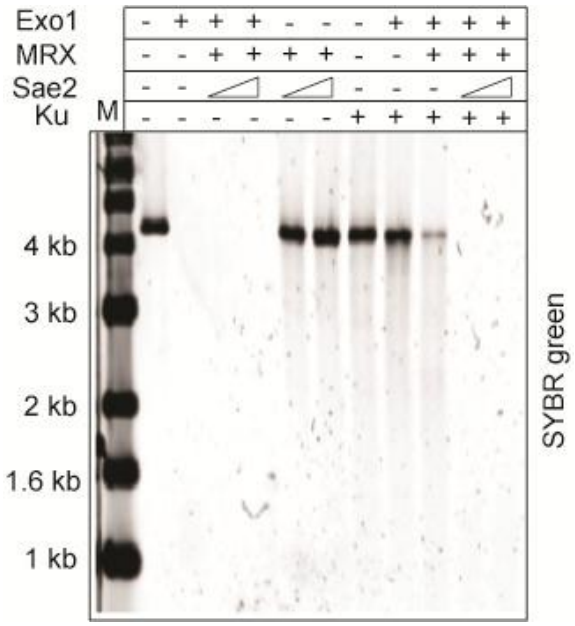


Figure 5.3 MRX and Sae2 cooperatively stimulate Exo1 exonuclease activity by inhibiting Ku.

Exo1 (1.4 nM), MRX (20 nM), Sae2 (20 or 40 nM), and Ku (7 nM) were tested in a resection assay as indicated above. Exonuclease products were resolved in a 1% agarose gel, and stained with SYBR green. Higher levels of Exo1, MRX, and Sae2 are used in this assay compared to the reaction shown in Figure 5.2 because of the inhibitory effect of Ku on Exo1 activity.

Mec1/Tel1 phosphorylation are essential for the role of Sae2 in DNA damage repair. In my study, I used recombinant Sae2 mutants purified from *E. coli*. Sae2 (S267A), an unphosphorylatable form and Sae2 (S267E), mimicking constitutively phosphorylated form showed similar levels of DNA binding ability and endonuclease activity *in vitro*. Sae2 (5D/S267E) was a little weaker in DNA binding and endonuclease activity by itself; however, in the presence of MRX, Sae2 (5D/S267E) binds equally well with DNA substrates, as compared to wild-type. Sae2 (5D/S267E) also showed a similar stimulation of Exo1 activity in resection assays compared to wild-type Sae2. Again the stimulation effect was a little weaker in the presence of Sae2 (5D/S267E), but together with MRX, they stimulate Exo1 activity significantly to a level similar to wild-type.

Genetic studies suggested that Ku inhibits Exo1 activity by limiting its access to DSB ends (Mimitou and Symington 2010; Shim, Chung et al. 2010). Deletion of *yKu* was observed to partially reverse the sensitivity of Δ*mre11* strains, and fully recover that of Δ*sae2* strains to DNA damaging reagents. Such a suppression effect was dependent on both *EXO1* and *SGS1*. These results suggest that MRX and Sae2 antagonize the inhibitory effect of Ku towards Exo1. My study *in vitro* using purified recombinant protein directly illustrates that both MRX and Sae2 play a role in the alleviation of the inhibitory effect of Ku toward Exo1 (Figure 5.3). In the presence of MRX, Sae2 (5D/S267E) stimulated Exo1 activity significantly better than wild-type, particularly in long-range resection (Figure 5.4, and 5.5). This could mean that the effect of Sae2 phosphorylation by CDK and Mec1/Tel1 is to regulate the choice of HR over NHEJ by limiting the inhibitory effect of Ku towards Exo1 during S and G_2 phase in response to DNA damage. However, Δ*yKu* did not suppress the MMS sensitivity I observed in *sae2 (S267A)* strains, while it did in Δ*sae2* strains (Figure 5.6). *In vivo* analysis suggests that

Sae2 phosphorylation may also play some other role in addition to relieving Ku inhibition.

Effect of phosphorylation on transition between multimeric forms

Recombinant Sae2 purified from *E. coli* was distributed in three peaks during size exclusion chromatography, which corresponds to three forms of Sae2: multimer, dimer, and monomer, as verified by sedimentation analysis (Lengsfeld, Rattray et al. 2007). One obvious paradox about Sae2 multimeric forms is that Sae2 mostly appears as aggregates in cells, however, the most active form *in vitro* is the monomer. The multimeric and dimeric forms are poor in DNA binding, and are barely active in the endonuclease assay or in stimulating Exo1 activity.

It is likely that in yeast, Sae2 transits from inactive multimers to active monomer form upon DNA damage, so as to assist in DSB end resection. This event is likely to occur during S and G_2 phase when Sae2 is needed to alleviate Ku inhibition towards Exo1, together with MRX. With recombinant Sae2 protein, I observed that Sae2 (5D/S267E) showed a two-fold increase in the percentage of the monomeric form in all Sae2 protein, as compared to wild-type (Figure 5.7). Both Sae2 (S267E) and Sae2 (5D/S267E) also show a significant decrease in the percentage of the multimeric form. Based on my *in vitro* analysis, CDK phosphorylation and Mec1/Tel1 phosphorylation could play a second role in regulation of DSB resection by converting more Sae2 to be in active monomeric form in response to DNA damage and during S and G_2 phase.

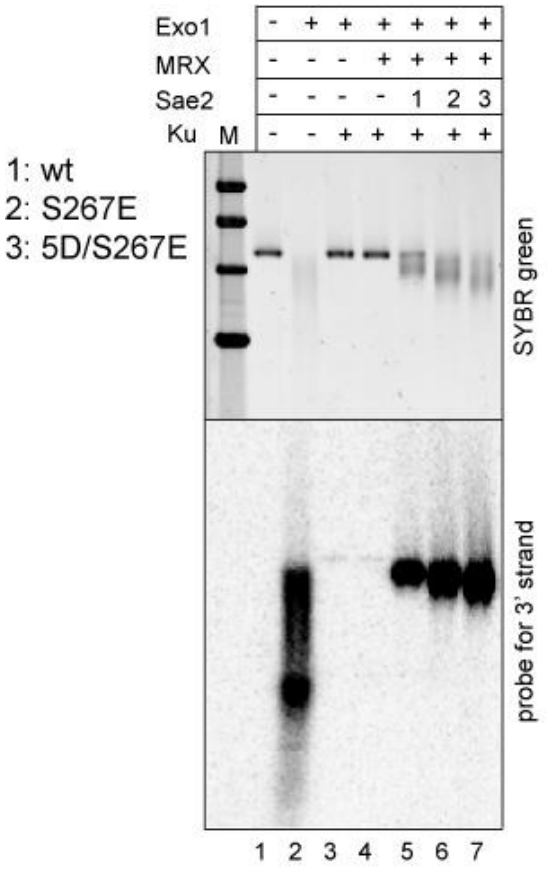


Figure 5.4 The effect of Sae2 phospho-mimic mutants on alleviation of Ku inhibition of Exo1-mediated resection

Exo1 (1 nM), MRX (10 nM), Sae2 (2.5 nM), and Ku (7 nM) were tested in resection assays with linearized pNO1 plasmid DNA substrate as in Figure 5.3 as indicated above. Exonuclease products were resolved in a 1% agarose gel, and stained with SYBR green (top panel). A non-denaturing southern blot was performed after SYBR green staining, and the membrane was probed with an RNA complementary to 3′ strand of the linearized pNO1 substrate in a 1 kb region adjacent to the *Sph*I site (bottom panel).

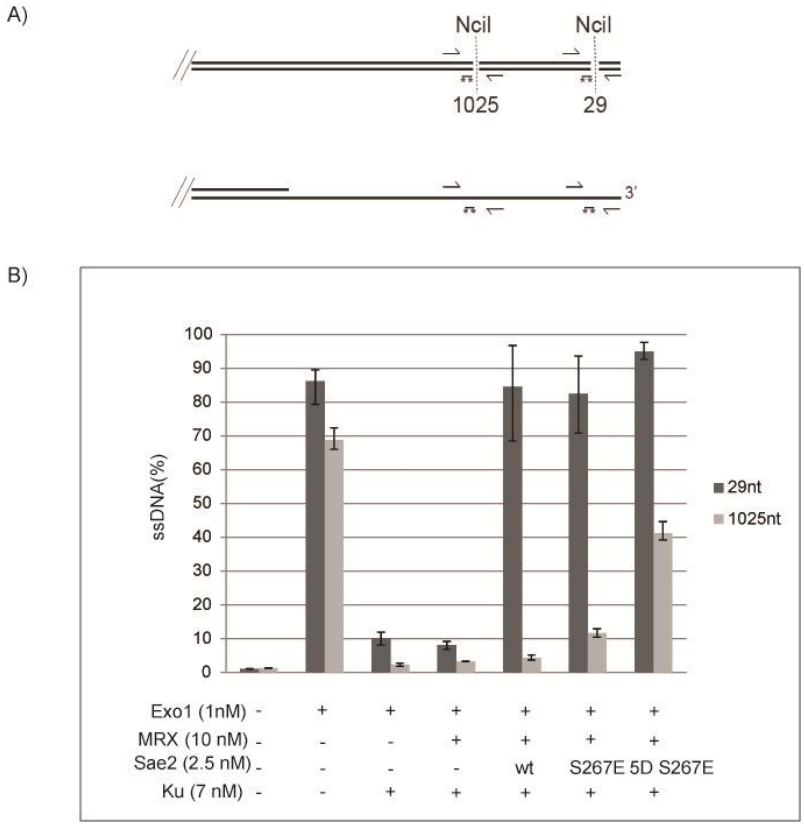


Figure 5.5 Quantitative measurement of ssDNA(%) after resection

A) Schematic diagram of quantitative PCR probes and NciI cleavage sites on one end of the plasmid DNA substrate. The end of pNO1 DNA substrate was cut with *Sph*I-HF. Two NciI sites are located 29 nt and 1025 nt from the cleavage site as shown. Locations of PCR primers (arrows) and qPCR 6-FAM-TAMRA probes (asterisks) are as labeled. NciI digests dsDNA (top) but leaves ssDNA intact (bottom). B) Two sets of primers, 29 or 1025 nt away from the 3' end of *Sph*I-HF cleavage on pNO1 plasmid, were used in quantitative PCR to measure the percentage of ssDNA at each site after the resection assay as performed in Figure 5.3. Three independent reactions were performed for each condition and the average was calculated, and the standard deviation is as shown.

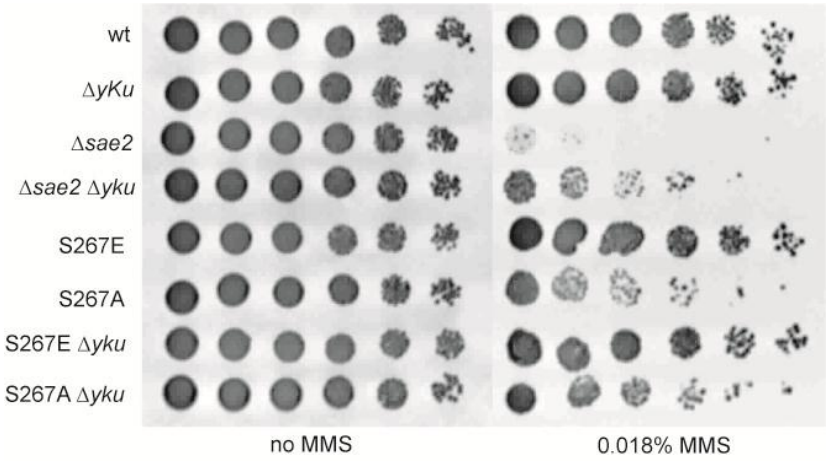
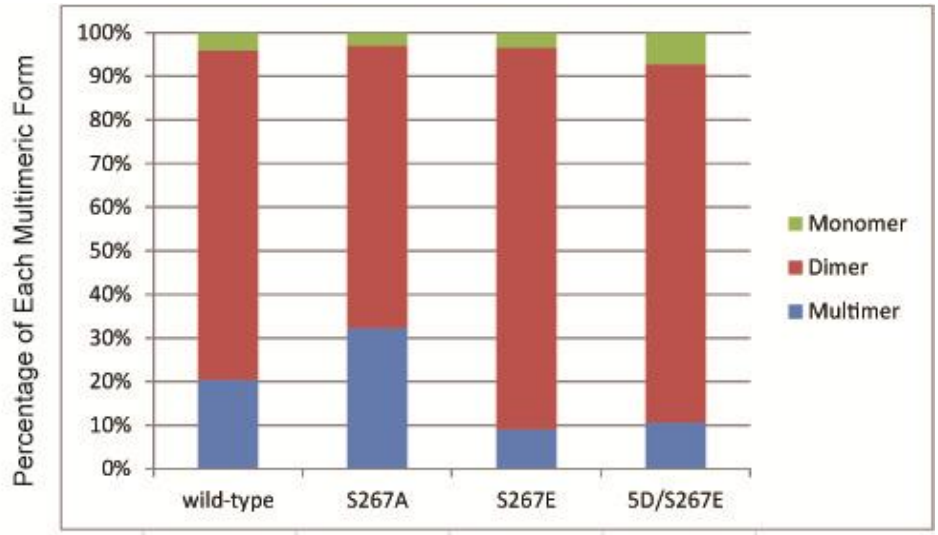


Figure 5.6 MMS sensitivity of various Sae2 and yKu mutants.

Yeast strains (isogenic to ALE94 (*MATa ade5-1 his7-2 leu2-3 112:p305L3(LEU2) trp1-289 ura3-D lys2:AluIR*), gift from K. Lobachev) with different genotypes as indicated were grown to the log phase, diluted to 1 O.D., 1/5 serial diluted, and spotted onto –LEU plates with (right) or without (left) MMS.



	wild-type	S267A	S267E	5D/S267E
Multimer	20.50%	32.34%	9.14%	10.70%
Dimer	75.41%	64.68%	87.52%	82.14%
Monomer	4.09%	2.98%	3.34%	7.16%

Figure 5.7 Distribution of three multimeric forms of recombinant Sae2

All Sae2 protein was purified using the same protocols as described in Methods and Materials. Purified recombinant Sae2 protein was applied to a Superdex 200 column, and distributed in three peaks corresponding to three multimeric forms. Protein amount were calculated using UV index. The percentage of each multimeric form of wild-type or mutant protein was calculated using the amount of each multimeric form divided by the total amount of each protein.

CHAPTER 6 DISCUSSION AND FUTURE DIRECTION

RECONSTITUTION OF THE SPO11 COMPLEX

Spo11 has long been suggested to be the catalyst of meiosis-specific DSB initiation (Esposito, Frink et al. 1972; Esposito and Esposito 1974; Neale, Pan et al. 2005; Keeney and Neale 2006). However, direct demonstration of Spo11 activity *in vitro* has been complicated by the insolubility of the Spo11 protein (Wu, Gao et al. 2004) as well as the essential requirement of at least nine other proteins (Rec102, Rec104, Ski8, Mer2, Mei4, Rec114) for DSB formation (Arora, Kee et al. 2004). In yeast, a large amount of Spo11 were found to be covalently attached to chromosomes in *rad50S*, *mre11S*, *mre11-58S*, and Δ*sae2* strains, which indicates a complete dependence on MRX and Sae2 to remove Spo11-DNA adducts (Alani, Padmore et al. 1990; Cao, Alani et al. 1990; Nairz and Klein 1997).

I successfully purified the core subcomplex (recombinant Spo11, Ski8, Rec102, and Rec104) for meiotic DSB initiation. Spo11 had either a Gal4BD-MBP tag or just an MBP tag at its N-terminus. Gal4BD-MBP-Spo11 was capable of binding DNA (Figure 3.3). Our preliminary analysis revealed that Gal4BD-MBP-Spo11 complex could perform cleavage on supercoiled plasmid DNA, and such activity was further stimulated by MRX and Sae2 (Figure 3.4). I purified a similar complex containing recombinant Spo11 (Y135F) mutant (Figure 3.2). Spo11 (Y135F) complex was functional in DNA binding but not in plasmid cleavage (Figure 3.3 and 3.4).

The occurrence of a small amount DSBs with the presence of Gal4BD-MBP-Spo11 only could be caused by the presence of Gal4BD-MBP tag in the N-terminus of Spo11. Although my initial goal was to reconstitute targeted Spo11 catalyzed DSB formation *in vitro*, Gal4BD-MBP-Spo11 complex was found to bind non-specifically to

DNA substrates, since its binding ability was not dependent on the presence of *GAL4* sites on the DNA. However, the binding between the MBP-Spo11 complex and either substrates either with or without *GAL4* sites was not as efficient (Figure 3.3). Probably, as a result of this deficiency in DNA-binding ability, the MBP-Spo11 complex was not active in plasmid cleavage. Thus the presence of GAL4BD-MBP tag at the N-terminus of Spo11 may help to overcome the requirement for other proteins (MRX, Sae2, Mer2/Mei4/Rec114) to make DSBs. We are now trying to purify the last complex, Mer2/Mei4/Rec114, essential for meiotic DSB formation *in vivo* (Arora, Kee et al. 2004). The Mer2/Mei4/Rec114 complex could function to assist the association between Spo11/Ski8/Rec102/Rec104 and hotspot DNA substrates, so as to activate Spo11. To verify this hypothesis and fully recapitulate the activity of Spo11, MRX and Sae2 observed in yeast, we plan to add Mer2/Mei4/Rec114 to the plasmid cleavage assay together with MBP-Spo11 complex, and to observe whether Spo11 catalyzed cleavage will occur and whether such activity will depend on the presence of MRX/Sae2 or not.

The difference between *in vivo* and *in vitro* analysis could also reflect the intrinsic different features of these two kinds of study. In cells, hotspots DNA is surrounded by nucleosome components. These components undergo highly-ordered and programmed rearrangements during meiosis. It is possible that some local and global changes of the chromatin structures around hotspots DNA cannot be mimicked accurately in my substrate *in vitro*. Besides, histone modifications may also regulate the accessibility of hotspots DNA. Understanding the difference between *in vivo* and *in vitro* experimental environment will help us to better interpret our results, as well as the discrepancy between the results.

STRUCTURAL FEATURES OF SAE2, A NOVEL ENDONUCLEASE

Sae2 was shown to be important for both meiotic and mitotic DNA repair *in vivo* (McKee and Kleckner 1997; Prinz, Amon et al. 1997). *In vitro*, Sae2 has endonuclease activity, but it is lacking any obvious typical nuclease domain (Lengsfeld, Rattray et al. 2007). Yeast Sae2 and its functional homolog CtIP in human differ significantly in their size, and they also only shares limited conserved residues at the C-terminus of the proteins, which was demonstrated to be important for CDK phosphorylation during S and G_2 phase (Huertas, Cortes-Ledesma et al. 2008).

I performed partial proteolysis on recombinant Sae2 protein (Figure 4.1), and analyzed various deletion and point mutants of Sae2 in three putative domains (N-terminal, central and C-terminal domains). The N-terminal domain (a.a. 1 – 174) is responsible for Sae2 dimerization (L25, ΔN), and Mec1/Tel1 phosphorylation (S84/E85/D86/F87). The central domain (a.a. 175 – 268) was active in endonuclease assay by itself in recombinant form *in vitro*. This domain contains many conserved residues, and they play an essential role in protein stability (D193, N197, P198, W204, D208) or DNA binding (I205, N212, E226). The C-terminal domain of Sae2 (a.a. 269 – 345) is the other conserved part of this protein. It is also a domain that is a target of many post-translational modifications. It was shown previously that the Sae2 (ΔC) (deletion of a.a. 250 - 345) mutant only had residual amount of endonuclease activity, and lost its cooperativity with MRX to cleave hairpin DNA (Lengsfeld, Rattray et al. 2007). It contains a stretch of amino acids important for DNA binding as well (G270D, D275A, E281A) (Table 4.1) (Lengsfeld, Rattray et al. 2007).

Sae2 appears in three forms after gel filtration column (multimer, dimer, and monomer). Monomeric form of Sae2 is so far the most active form we observed in all

assays *in vitro*, however, in yeast, Sae2 mostly appears in multimeric form (Kim, Vijayakumar et al. 2008)(Qiong Fu, personal communication). My study and previous results (Lengsfeld, Rattray et al. 2007) indicated that two parts (L25 & a.a. 120 - 170) in the N-terminal domain are responsible for Sae2 dimerization. Little is known about the transition of Sae2 from inactive multimers to active monomer under physiological conditions, and the importance of dimerization. Interestingly, Sae2 (L25P) behaves normally in the endonuclease assay *in vitro*, but completely fails to process hairpin intermediates *in vivo* (Table 4.1). The multimer and dimer form of Sae2 are very likely to serve as a "reservoir" of inactive Sae2. In response to DNA damage and during proper cell cycle stages, Sae2 undergoes certain modifications. It may change its conformation, and more active monomeric form of Sae2 may get released from the "reservoir" to participate in DSB end resection.

There are five putative Mec1/Tel1 phosphorylation sites in Sae2 (Baroni, Viscardi et al. 2004). Although whether they are truly targets of Mec1/Tel1 in response to DNA damage awaits to be verified, it is interesting that two of them are in the non-conserved N-terminal part, while the rest are in the conserved C-terminal part of Sae2. This could suggest some interaction between N-terminus and C-terminus of Sae2. Alternatively, it is also possible that the multimer/dimer of Sae2 is in a head-to-tail form (N to C, and C to N), so that phosphorylation by Mec1/Tel1 could change the conformation of multimer by disrupting the interaction between N and C termini, so as to release active monomeric form of Sae2.

Sae2 has endonuclease activity *in vitro* (Lengsfeld, Rattray et al. 2007). However, it remains a challenge to characterize catalytic residue (s) for its endonuclease activity. I targeted many acidic residues in the central domain, since the purified recombinant form

of this central domain alone showed endonuclease activity. I found a double mutant Sae2 (E207A/D208Q) that showed a 5-fold decrease in endonuclease activity (Figure 4.3), but it stills had protein instability problems *in vivo*. Since there is more than one part of Sae2 responsible for DNA binding (part of both central domain and C-terminus), it is possible that Sae2, as non-canonical endonuclease, also has more than one region that is essential for catalytic activity. This suggests some potential functional interaction between central domain and C-terminus to perform endonuclease cleavage. To identify the active sites of Sae2 and construct a completely nuclease deficient mutant, we plan to point mutate more residues around DNA-binding deficient region in the central and C-terminal domains.

PHOSPHORYLATION OF SAE2 PLAYS A DUAL ROLE IN REGULATION BETWEEN HR AND NHEJ

HR and NHEJ are highly regulated molecular events. In yeast, HR is more dominant in S and G_2 phase when there is a second copy of DNA content available. The switching between HR and NHEJ is governed by CDK. Among proteins responsible for DSB resection and HR, Sae2 and Dna2 are the only two enzymes known so far regulated by CDK. Dna2 was regulated at a transcriptional level (Huertas, Cortes-Ledesma et al. 2008; Kosugi, Hasebe et al. 2009). Sae2 is phosphorylated by CDK at S267 during S and G_2 phase. In response to DNA damage, Sae2 is also a target of phosphorylation by Mec1/Tel1 (Baroni, Viscardi et al. 2004). Both modification events are important for Sae2 function in DNA repair, since disruption of either CDK site or Mec1/Tel1 sites in Sae2 cause hypersensitivity to DNA damage reagents in yeast (Huertas, Cortes-Ledesma et al. 2008).

Using recombinant proteins *in vitro*, I found that Sae2 (5D/S267E) mimicking constitutive phosphorylation, with MRX, is more efficient in relieving Ku inhibition

towards Exo1 (Figure 5.4 and 5.5). This increase in Exo1 activity is specific to the presence of Ku. In the absence of Ku, MRX and Sae2 (5D/S267E) only stimulate Exo1 activity to a level similar to MRX and wild-type Sae2 (Figure 5.2). Yet Δ*yKu* cannot fully recover the deficiency of strains expressing Sae2 (S267A) in MMS sensitivity assay, as it did for Δ*sae2* strains (Figure 5.6). I partially explained this discrepancy by analyzing the distribution of three forms (multimer, dimer, and monomer) of recombinant Sae2 (Figure 5.7). Compared to wild-type, Sae2 (S267A) showed more multimer form, while

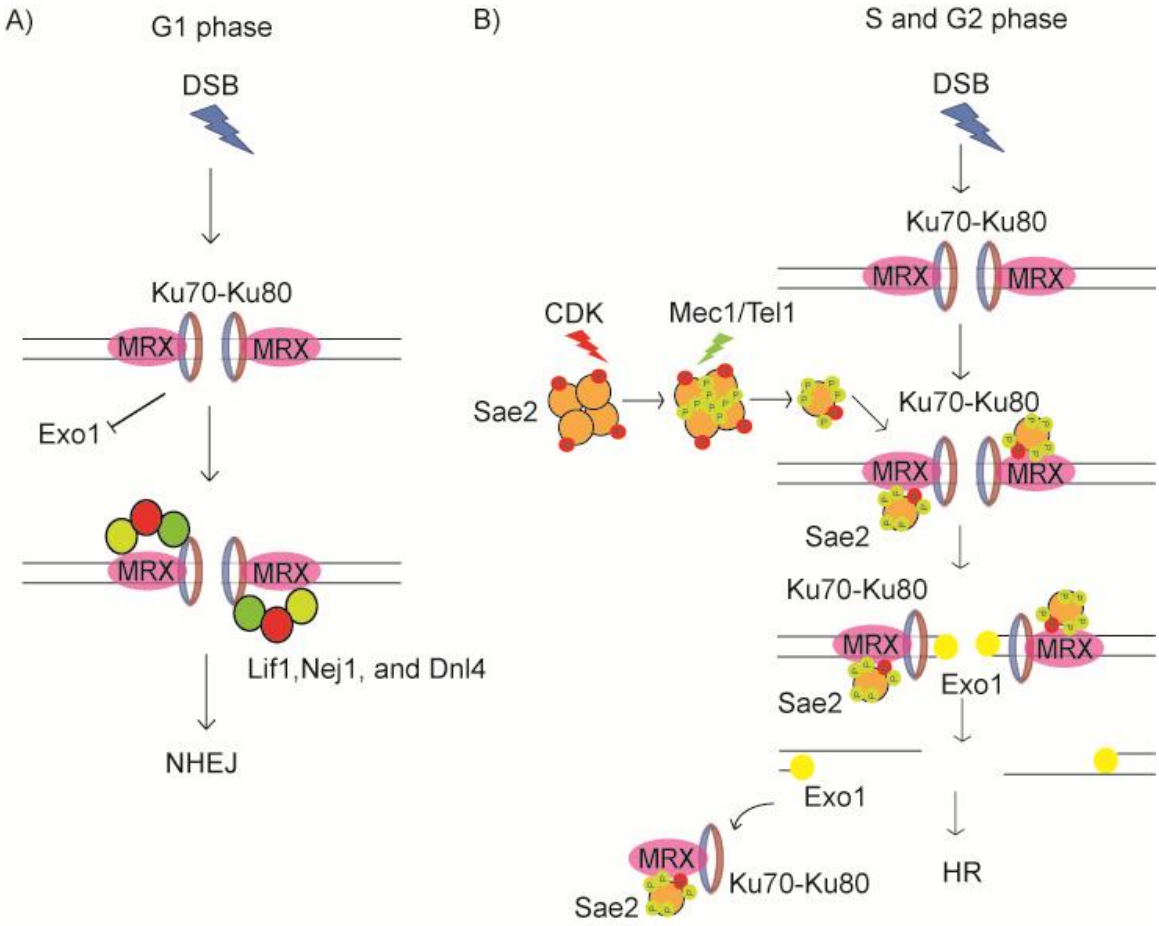


Figure 6.1 Models for the interplay between Ku, MRX and Sae2

A) In G_1 phase, in response to DNA damage, Ku and MRX are recruited to DSB ends. Ku and MRX both participate in recruiting Lif1, Nej1 and Dnl4 important for NHEJ repair pathway. Ku also blocks the accessibility of Exo1 to DSB ends, so as to block HR. B) In S and G_2 phase, after DNA damage, active monomeric Sae2 is released upon CDK phosphorylation and Mec1/Tel1 phosphorylation. Phosphorylated Sae2 is more efficient in relieving the inhibition of Ku towards Exo1. Exo1 binds and processes DSB ends to generate 3' single-stranded DNA overhang, and commit cells to HR repair pathway.

Sae2 (S267E) and Sae2 (5D/S267E) showed more dimer form, and Sae2 (5D/S267E) had a two-fold increase in the monomeric form of Sae2 (Figure 5.7). Thus, the phosphorylation of Sae2 by CDK and Mec1/Tel1 could play a dual role in regulation between HR and NHEJ, by making Sae2 more competent in repelling Ku inhibition towards Exo1, and by freeing more Sae2 to be in active monomer form. To further verify the transition of Sae2 to monomer form upon DNA damage, we are immunoprecipitating and analyzing Sae2 from yeast cells treated with DNA damaging reagents. To understand how Sae2 (5D/S267E) relieves Ku inhibition towards Exo1 better, we plan to analyze the location of Ku in the presence of MRX and Sae2 to observe whether Ku is pushed further away from DSB ends, or completely removed from DSB ends by MRX and Sae2. Understanding the dynamic interaction between Ku, MRX and Sae2 will help to clarify the mechanism of switching between HR and NHEJ during the cell cycle in response to DNA damage.

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